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(54) Title: METHODS FOR THE IDENTIFICATION OF AGENTS FOR THE TREATMENT OF SEIZURES, NEUROLOGICAL DISEASES, ENDOCRINOPATHIES AND HORMONAL DISEASES

(57) Abstract: The present invention is drawn to methods of characterization of the properties and functions of SV2 proteins. The invention further includes methods of identifying compounds or agents which modulate the activity of SV2 proteins. Included in these methods is the identification of compounds or agents which modulate the binding of levetiracetam to SV2 proteins, including SV2A. Additionally, the present invention provides biotinylated ligands as a tool to screen chemical libraries and characterize the SV2 proteins. Further, the present invention provides a method of solubilizing and purifying functionally active membrane associated proteins, such as SV2.

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**METHODS FOR THE IDENTIFICATION OF AGENTS FOR THE TREATMENT
OF SEIZURES, NEUROLOGICAL DISEASES, ENDOCRINOPATHIES AND
HORMONAL DISEASES**

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RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application 60/506,764, filed September 30, 2003, and U.S. Provisional Application 60/430,372, filed December 3, 2002,
10 which are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention is generally drawn to the field of drug discovery in neurological disorders, endocrinopathies and hormonal diseases.

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BACKGROUND OF THE INVENTION

Neurological disorders afflict a substantial number of individuals and present an increasing economic challenge to health care systems since little is known regarding their causes, their diagnosis is often subjective, and many lack effective treatment. In general,
20 brain activity is ultimately determined by the capacity of neurons to communicate at synapses. Specific neurotransmitter chemicals are packaged in presynaptic neurons into synaptic vesicles which fuse with the presynaptic membrane to release *quanta* of the neurotransmitter chemical that traverse the synaptic cleft to activate the corresponding receptor type resident in the post-synaptic membrane. Among these receptor types are the
25 neuronal glutamate receptors (GluR's), γ -aminobutyric acid receptors (GABAR's), nicotinic acetylcholine receptors, serotonin receptors, dopamine receptors, and the like. Many neurological disorders are a result of improper conduction of electrical currents through synapses in various brain tissues. In epilepsy errant currents, hypothesized to be associated with improper function of synapses, cause various levels of seizures. Likewise, in several
30 psychiatric diseases, movement disorders and neurodegenerative diseases the conduction currents become aberrant, disorganized or reduced, thereby causing the disease condition.

Accordingly, defects in synaptic vesicle functions will have an adverse effect upon neurotransmission in general and control of neurotransmitter release in particular.

Seizures, including epileptic seizures, result from a focal or generalized disturbance of cortical function, which may be due to various cerebral or systemic disorders, including, for example, cerebral edema, cerebral hypoxia, cerebral trauma, central nervous system (CNS) infections, congenital or developmental brain defects, expanding brain lesions, hyperpyrexia, metabolic disturbances and the use of convulsive or toxic drugs. It is only when seizures recur at sporadic intervals and over the course of years (or indefinitely) that epilepsy is diagnosed.

Epilepsy is classified etiologically as symptomatic or idiopathic with seizure manifestations that fall into three general categories: 1) generalized tonic-clonic, 2) absence or petiti mal, and 3) complex partial. Symptomatic classification indicates that a probable cause exists and a specific course of therapy to eliminate that cause may be tried, whereas idiopathic indicates that no obvious cause can be found and may be linked to unexplained genetic factors. Of the seizure categories, most persons have only one type of seizure, while about 30% have two or more types.

The risk of developing epilepsy is 1% from birth to age 20 yr. and 3% at age 75 yr. Idiopathic epilepsy generally begins between ages 2 and 14. Seizures before age 2 are usually caused by developmental defects, birth injuries, or a metabolic disease. Those beginning after age 25 may be secondary to cerebral trauma, tumors, or cerebrovascular disease, but 50% are of unknown etiology.

Due to the many interrelationships that exist between the nervous and endocrine systems, defects in synaptic vesicle function can also impact on endocrinological function. For instance, at least two glands secrete their hormones only in response to appropriate neurotransmitter release - the adrenal medulla and the posterior pituitary gland. Upon secretion, hormones are transported in the blood to cause physiologic actions at distant target tissues in the body. Obviously, endocrinopathies involving either hyper- or hyposecretion of hormones have pathological consequences. Exemplary of these consequences are gigantism and dwarfism, due to hyper- or hyposecretion of growth hormone, respectfully.

Levetiracetam

Levetiracetam (LEV; ucb L059; (S)- α -ethyl-oxo-pyrrolidine acetamide), the (S)-enantiomer of the ethyl analog of piracetam, was synthesized during a follow-up chemical program aimed at identifying a second-generation nootropic drug. *In vivo* results have demonstrated an unexpected potent ability of LEV to suppress seizures in the audiogenic-susceptible mouse, whereas piracetam was only weakly active. Although LEV is a molecule unrelated to established antiepileptic drugs (Margineanu *et al.*, in Antiepileptic Drugs: 5th Edition. pp. 419-427. Lippincott, Philadelphia (2002)), extensive clinical trials have proven that adjunctive therapy with LEV (KEPPRA, UCB, S.A., Braine-l'Allend, Belgium) is both effective and well tolerated in controlling refractory partial seizures in adults.

Binding assays with LEV, performed on crude rat brain membranes, reveal the existence of a reversible, saturable and stereoselective specific binding site. Results obtained in rat hippocampal membranes suggest that LEV labels a single class of binding sites with modest affinity and with a high binding capacity. This binding site is identified as the Levetiracetam Binding Site (LBS). Similar results have been obtained in other brain regions (cortex, cerebellum and striatum). Ucb L060, the (R)-enantiomer of levetiracetam, displays about 1000 times less affinity for these sites. The binding of LEV appears to be confined to membranes in the central nervous system since radiolabel studies could detect no specific binding in a range of peripheral tissues including heart, kidneys, spleen, pancreas, adrenals, lungs and liver. However, this could be due to a low density of LBS in these tissues compared to the central nervous system and indeed specific binding does occur in PC12 cells, a peripherally derived adrenal cell line.

The most commonly used antiepileptic drugs carbamazepine, phenytoin, valproate, felbamate, gabapentin, tiagabine, vigabatrin, zonisamide, phenobarbital and clonazepam, as well as the convulsant agent t-butylbicyclophosphorothionate (TBPS), picrotoxin and bicuculline do not displace LEV binding (Gillard *et al.* Eor. J. Pharmacol. 478:1-9. (2003)). However, ethosuximide, pentobarbital, pentylenetetrazole and bemegride competed with LEV with pKi values comparable to active drug concentrations observed *in vivo*. Structurally related compounds, including piracetam and aniracetam, also displaced LEV binding. The levetiracetam analogues were also tested for their anticonvulsant activity in the audiogenic mouse model of epilepsy. A very good correlation ($r^2=0.84$) was observed between the

affinity and the anticonvulsant activity (Noyer *et al.*, Euro. J. Pharmacol. 286:137-146. (1995)). This high degree of correlation is strong support for a causative relationship between LBS binding and anticonvulsant activity of this class of compounds. Accordingly, binding of levetiracetam analogues to LBS is expected to result in modification of the function of the protein component(s) of the LBS in brain, leading to the desired therapeutic outcome of anticonvulsant activity.

The Synaptic Vesicle Protein 2 Family

The Synaptic Vesicle Protein 2 (SV2) family of synaptic vesicle proteins was first identified with a monoclonal antibody prepared against cholinergic vesicles from the electric organ of the marine ray *D. ommata* (Buckley *et al.*, J. Cell Biol. 100:1284-1294. (1985)). Cloning of the individual family members labeled by the antibody resulted in the identification of three different isoforms, SV2A (Bajjalieh *et al.*, Science. 257:1271-1273. (1992)), SV2B (Feany *et al.*, Cell. 70(5):861-867. 1992) and SV2C (Janz and Sudhof, Neuroscience 94(4): 1279-1290. (1999)), all of which react with the original antibody. The overall homology between the three rat isoforms is approximately 60%, with SV2A and SV2C being more similar to each other than SV2B (Janz and Sudhof, Neuroscience 94(4): 1279-1290. (1999)).

The SV2 proteins are integral membrane proteins and have significant but low-level homology (20-30%) to the twelve transmembrane family of bacterial and fungal transporter proteins that transport sugar, citrate, and xenobiotics (Bajjalieh *et al.*, Science. 257:1271-1273. (1992)). As putative members of the 12 TM superfamily, SV2 proteins display several unique features. They have relatively short free N- and C- termini and short loops connecting the Tm segments. Two notable exceptions, however, are the long cytoplasmic loop between transmembrane regions 6 and 7 and the intravesicular loop between transmembrane regions 7 and 8 (which contains 3 N-glycosylation sites). No close homologs of the SV2 proteins have yet been discovered in yeast or invertebrates, although a distantly related synaptic vesicle protein known as SVOP does have homologs in *Drosophila* and *C. elegans* (Janz *et al.*, J. Neurosci. 18(22):9269-9281. (1998)).

As a family, SV2 proteins are widely distributed in the brain and in endocrine cells. The three isoforms overlap significantly in their distribution, and can be found co-expressed

in the same neuron, and even on the same synaptic vesicle. One isoform or another of the SV2 proteins seems to be present on all synaptic vesicles, and they are probably not limited to neurons that contain any specific neurotransmitters, although one study reports that cholinergic vesicles may not contain SV2 (Blumberg *et al.*, J. Neurochem. 58(3):801-810 (1992)). SV2 proteins are therefore one of the most common proteins of synaptic vesicles, and have been implicated in the control of calcium-mediated exocytosis of synaptic vesicles. SV2 proteins have also been shown to be expressed in endocrine cells and, along with the additional synaptic vesicle membrane integral proteins p38 and p65, has been demonstrated to be present in endocrine dense core granule membranes (Lowe *et al.*, J. Cell. Biol. 106(1):51-59 (1988). SV2A, the most common SV2 isoform, is expressed ubiquitously throughout the brain, and is present as well in secretory granules of endocrine cells. SV2B, while broadly distributed in the brain, is undetected in several brain structures, including the dentate gyrus of the hippocampus, the globus pallidus, reticular nuclei of the thalamus, and the reticular part of the substantia nigra (Bajjalieh *et al.*, 1994). By contrast, SV2C has quite a limited distribution and is found primarily the phylogenetically old regions such as the pallidum, the substantia nigra, the midbrain, the brainstem and the olfactory bulb. It is undetectable in the cerebral cortex and the hippocampus, and found at low levels in the cerebellar cortex (Janz and Sudhof, Neuroscience 94(4): 1279-1290. (1999)).

In addition to the SV2 protein, the synapse contains other unique regulatory proteins such as synapsin, synaptotagmin and CAPS, which may mediate vesicle fusion or budding. SV2A may be a Ca^{2+} regulatory protein essential for the formation of pre-fusion complexes called SNARE complexes (Xu *et al.* Cell 99(7):713-722 (1999)), which include the synaptic vesicle-associated VAMP/synaptobrevin and the plasma membrane proteins syntaxin and SNAP-25. Upon Ca^{2+} accumulation in the synapse the binding of synaptotagmin to SV2A is inhibited and the dimerization of two synaptotagmin Ca^{2+} binding domains is stimulated (Bajjalieh, Curr. Opin. Neurobiol. 9(3):321-328. (1999)). This dimerization may play a role in organizing the SNARE complex and promoting vesicle fusion, as at low Ca^{2+} concentrations, SV2A remains bound to synaptotagmin and fusion will not occur.

The affinity of SV2A for synaptotagmin is regulated by the phosphorylation of the amino terminus of SV2 (Pyle *et al.*, J. Biol. Chem. 275(22):17195-17200. (2000)). The possibility that SV2 proteins play a role in either Ca^{2+} transport, or regulation in the synaptic

vesicle has been supported by studies of SV2A and SV2B knockout animals (Janz *et al.*, Neuron 24:1003-1016. (1999)). An alternative hypothesis is that the SV2 proteins, while derived from transport proteins, now serve a different function in the vesicle, whether a structural role or a role in regulation of vesicle fusion or recycling and the exocytotic release of their contents (Janz and Sudhof, Neuroscience 94(4): 1279-1290. (1999)).

There have been two reports of SV2 protein knockout mice: one that examines only SV2A knockouts (Crowder *et al.*, Proc. Nat. Acad. Sci. USA 96(26):15268-15273. (1999)) and the other which looks at both SV2A and SV2B knockout animals, as well as the SV2A/SV2B double knockout (Janz *et al.*, Neuron 24:1003-1016. (1999)).

Animals homozygous for SV2A gene disruption appear normal at birth, but fail to grow, experience severe seizures, and die within the first few weeks postnatal. SV2A homozygous knockout mice experience seizures that are longer lasting, stronger, and more debilitating than any other mouse strain (Janz *et al.*, Neuron 24:1003-1016. (1999)). Despite the appearance of postnatal seizures, all SV2A knockout animals have completely normal gross brain morphology, including normal levels of the tested synaptic proteins.

Furthermore, the hippocampal neuronal cultures from both SV2A and SV2A/SV2B double knockout mice formed synapses that were ultrastructurally normal, and had unchanged size, number and location of synaptic vesicles (Janz *et al.*, Neuron 24:1003-1016. (1999); Crowder *et al.*, Proc. Nat. Acad. Sci. USA 96(26):15268-15273. (1999)). Unlike the frequently

observed seizures caused by structural and developmental abnormalities easily detected in many other type of knockouts, the SV2A knockout mice show a strong seizure phenotype with no associated macro or micro scale abnormalities of the brain or synapse. As another marker of brain function, studies of synaptic transmission in primary neuronal cultures from SV2A, SV2B, and SV2A/SV2B knockout mice indicate that the sizes and frequencies of sIPSCs and of spontaneous excitatory postsynaptic currents (sEPSCs), are normal. Electrical stimulation induced robust EPSCs and IPSCs in the cultured neurons from all genotypes.

In contrast to SV2A, SV2B knockout mice reveal no overt pathology (Janz *et al.*, 1999). It is suggested that one possible reason for this lack of consequence of loss of SV2B is that can be functionally replaced by SV2A, which appears to be co-expressed everywhere SV2B is normally expressed.

While the function of SV2A and other family members still remains unknown, one

hypothesis is that this transporter homologue is a functional transporter for some common synaptic vesicle molecule. More specifically, there is evidence linking SV2A to the regulation of calcium-mediated vesicle exocytosis, and as a result, it is thought that it may be a Ca^{2+} transporter. SV2A and other family members may also have roles in the function of synaptic vesicles. Such roles may include modulating aspects of their formation, loading with neurotransmitter, fusion with the plasma membrane, re-cycling, and interactions with other proteins and cellular compartments and organelles. For instance it has been shown that SV2 proteins can interact with the synaptic vesicle protein synaptotagmin and the extracellular matrix protein laminin-1 (Carlson, Perspect. Dev. Neurobiol. 3(4):373-386 (1996)). The SV2 proteins may play important roles in regulating cytoplasmic or organellar calcium levels at the presynaptic terminal, and may also interact with N-type calcium channels on the plasma membrane, either directly or indirectly.

SUMMARY OF THE INVENTION

The present inventors have discovered that SV2A is the binding site for the anti-seizure drug LEV and its analogs. The high degree of correlation between relative binding affinities of a series of levetiracetam analogues and their anti-convulsant potencies in certain animal models of epilepsy provides strong evidence that binding of these analogues to SV2 proteins modifies their function to provide anticonvulsant effects.

In a preferred embodiment, the invention includes a method of treating a neurological disorder associated with synaptic vesicle function, endocrinopathy or hormonal diseases, comprising administering a compound or agent that modulates a function or activity of an SV2 protein.

In another preferred embodiment, the invention includes a method of discovering or modeling an interaction between an SV2 protein and a compound or agent selected from the group consisting of: levetiracetam, an analog or derivative of levetiracetam, or a compound or agent which competes with levetiracetam or an analog or derivative thereof for binding to the levetiracetam binding site. The method comprises contacting the SV2 protein with the compound or agent measuring and analyzing the interaction of the SV2 protein with the compound or agent.

In another preferred embodiment, the invention includes a method of identifying a

levetiracetam binding site within an SV2 protein. The method comprises contacting a SV2 protein or fragment thereof with a compound or agent selected from the group consisting of levetiracetam, an analog or derivative of levetiracetam, or a compound or agent which competes with levetiracetam or an analog or derivative thereof for binding to the
5 levetiracetam binding site and determining the binding of the compound or agent with the SV2 protein or fragment thereof.

In another preferred embodiment, the invention includes a method of assaying the interaction between SV2 protein and a second protein. The method comprises expressing SV2 protein and the protein of interest in a cell. The method further comprises exposing the
10 cell to a compound or agent which binds to the levetiracetam binding site and determining the interaction between the SV2 protein and the protein of interest.

In another preferred embodiment, the invention includes a method of identifying a compound or agent that modulates a neurological disorder associated with synaptic function, endocrinopathy or hormonal disease. The method comprises exposing a SV2 protein to the
15 compound or agent and determining whether the compound or agent modulates an activity of the SV2 protein.

In another preferred embodiment, the invention includes a method of identifying a cellular response to a compound or agent selected from the group consisting of levetiracetam, an analog or derivative of levetiracetam, or a compound or agent which competes with
20 levetiracetam or an analog or derivative thereof for binding to the levetiracetam binding site. The method comprises exposing cells expressing an SV2 protein to the compound or agent and analyzing a change in the expression of a nucleic acid or protein in the exposed cell. The nucleic acid could be RNA, and the expression of the RNA may be analyzed by hybridization, such as hybridization on a microarray.

25 In another preferred embodiment, the invention includes an isolated nucleic acid molecule comprising the nucleic acid sequence of SEQ ID NO: 5 or the complement thereof.

In another preferred embodiment, the invention includes a method of identifying a binding partner for a SV2 protein. The method comprises exposing a SV2 protein or fragment to a potential binding partner and incubating the protein or fragment and potential
30 binding partner with (2S)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide. The method further comprises determining if the binding of (2S)-2-[4-(3-azidophenyl)-2-

oxopyrrolidin-1-yl]butanamide to the protein is inhibited by the potential binding partner, thereby identifying binding partner for the protein.

In still another preferred embodiment, the invention includes a method of identifying an agent useful for the treatment of a neurological or endocrinological disorder. The method
5 comprises exposing a SV2 protein or fragment to the agent and levetiracetam or an analog or derivative thereof. The method further comprises determining if the binding of levetiracetam or an analog or derivative thereof to the protein is modulated by the agent, thereby identifying an agent useful for the treatment of a neurological or endocrinological disorder.

In yet another preferred embodiment, the invention includes a method of identifying
10 an agent useful for the treatment of a neurological or endocrinological disorder. The method comprises exposing a SV2 protein or fragment to the agent and incubating the protein or fragment and agent with (2S)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide. The method further comprises determining if the binding of (2S)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide to the protein is inhibited by the agent, thereby identifying
15 binding partners for the protein.

In another preferred embodiment, the invention includes a method of discovering or modeling an interaction between an SV2 protein, or fragment or derivative thereof, and a compound or agent selected from the group consisting of: levetiracetam, an analog or derivative of levetiracetam, or a compound or agent which competes with levetiracetam or an
20 analog or derivative thereof for binding to the levetiracetam binding site. The method comprises creating a 3-dimensional model of the SV2 protein, or fragments thereof, via either biochemical, biophysical, purely computational techniques, or some combination of these and creating 3-dimensional model of one or a collection of potential ligands that might potentially bind the SV2 protein.

25 In another preferred embodiment, the invention includes a method of discovering or modeling an interaction between an SV2 protein and a compound or agent selected from the group consisting of: levetiracetam, an analog or derivative of levetiracetam, or a compound or agent which competes with levetiracetam or an analog or derivative thereof for binding to the levetiracetam binding site. The method comprises determining a biochemical,
30 pharmacological, organismal, cellular or molecular effect of a potential CNS active molecule in a genetically wild-type animal or in molecules, cells or tissues derived from such animals

and comparing the measured effect of that compound in an equivalent study in a system with an SV2 protein knocked out or knocked down.

The present invention also provides biotinylated ligands as tools to screen chemical libraries, localize SV2 proteins in tissues, and characterize purified SV2 proteins. SV2 proteins of the present invention includes SV2A, SV2B, and SV2C. Ligands of SV2/LBS, specifically SV2A/LBS, and their derivatives may be biotinylated for screening naturally occurring brain membranes, such as animal, mammalian, or human brain membranes, or for screening cell lines expressing SV2 proteins. The present invention also provides photoactivable biotinylated ligands of SV2/LBS. These screening assays enable the identification of new drugs or compounds that interact with SV2.

Further, the present invention provides a method of purifying a membrane associated protein comprising solubilizing the protein from a tissue to form a solubilized complex and isolating the solubilized complex in a functional form. The solubilized protein or complex may be affinity purified using antibodies that bind to the protein. Examples of membrane associated proteins that may be purified by this method include the family of SV2 proteins such as SV2A, SV2B, and SV2C. The detergents that may be used in the present method includes n-dodecyl- β -maltoside and its analogs or derivatives such as n-octyl, n-nonyl, n-decyl, n-undecyl- β -D-maltoside.

The biotinylated ligands also can be used as tools to assess the conformation state of SV2 proteins after solubilization, immunoaffinity purification, and chromatography.

In one embodiment, the SV2 protein may be a fusion protein comprising at least one SV2 protein or fragment thereof and fusion partner. The fusion partner may be a fusion tag, such as a poly-histidine tag or a glutathione-S-transferase tag. The fusion partner may be attached to the N-terminus or the C-terminus of the SV2 protein.

In another embodiment, the protein, such as the SV2 protein, may lack at least one glycosylation site. In some instances, site-directed mutagenesis may be performed to remove one or more glycosylation site in the SV2 protein.

The SV2 protein or fragment may be purified from natural sources such as mammalian membranes, for example, rat brain membrane. Alternatively, the SV2 protein or fragment is expressed on a transformed host cell. Additionally, the SV2 protein or fragment is immobilized.

In one aspect, the ligand could be directly or indirectly labeled. The label could be a radiolabel, such as ^3H , a fluorescent label, or an enzyme.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 depicts the reversible binding of the LEV analog ucb 30889 to LBS in rat brain cortex.

Figure 2 depicts the saturation binding curves of ucb 30889.

Figure 3 shows that specific binding could not be detected in the peripheral tissues.

10 Figure 4 depicts competition binding curves showing that ucb 30889 binds to LBS with about 10 fold higher affinity than LEV.

Figure 5 depicts pIC_{50} values for ucb 30889 versus levetiracetam.

Figure 6 depicts the concentration dependent inhibition of [^3H]ucb 30889 binding by unlabeled levetiracetam in autoradiography of rat brain.

15 Figure 7 depicts autoradiography of [^3H]ucb 30889 binding to coronal sections of rat brain.

Figure 8 depicts the subcellular distribution of [^3H]ucb 30889 binding within rat brain.

Figure 9 depicts the subfractionation of the synaptosomal fraction by centrifugation in sucrose gradient.

20 Figure 10 depicts the photolabelling of the LBS by [^3H]ucb 30889 and irreversibility of the complex.

Figure 11 depicts gel electrophoresis of membrane proteins labeled by [^3H]ucb 30889.

25 Figure 12 (A and B) depicts immunostained lysates of the COS-7 cells transfected with SV2A, crude rat brain membranes, and several different PC12 lysates with different levels of LBS.

Figure 13 depicts specific binding of [^3H]ucb 30889 to COS-7 transfected with SV2A-12.2, transfected with control β -gal expressing vector, or cells that have not been transfected.

30 Figure 14 depicts an IC_{50} plot comparing three different ligands binding to SV2A in the presence of 3H-30889.

Figure 15 depicts the structure of (A) levetiracetam and (B) ucb 30889.

Figure 16 (A and B) depicts binding of [3 H]ucb 30889 to brain membranes. A. Binding of [3 H]ucb 30889 to brain membranes from SV2A, SV2B, and SV2A/SV2B knockout mice. [3 H]ucb 30889 alone (□) [3 H]ucb 30889 plus 1mM LEV (■). Error bars are the SD of experiments performed with 5 wildtype brains and 4 KO brains. Each experiment was performed in triplicate. B. Western blot of brain membranes from wild type and homozygous knockout mice probed with an anti-SV2 monoclonal antibody (cross-reactive to all isoforms, SV2A, SV2B and SV2C). LANES 1: wt; 2: SV2A ko; 3 SV2B ko; 4: SV2A/B double ko.

Figure 17 (A and B) depicts binding of [3 H]ucb 30889 to COS-7 cells expressing hSV2A. A. Binding of [3 H]ucb 30889 to hSV2A transiently expressed in COS-7 cells. [3 H]ucb 30889 is tested for binding to either untransfected COS-7 cells, or COS-7 cells transiently expressing either \square -gal or hSV2A. [3 H]ucb 30889 alone (□) [3 H]ucb 30889 plus 1mM LEV (■). B. IC₅₀ curves of LEV, ucb L060, ucb 30889 against hSV2A transiently expressed in COS-7, in the presence of [3 H]ucb 30889. LEV (Δ) ucb 30889 (■) ucb L060 (\bullet). Error bars are SEM, n=3.

Figure 18 (A and B) depicts binding of [3 H]ucb 30889 in the presence of competing drugs. A. Correlation of binding of a series of LEV compounds to mouse brain and to hSV2A, pIC₅₀s measured against [3 H]ucb 30889. The pIC₅₀ values are the mean of two independent experiments, where each determination lies within 0.2 log units of the mean. B. Correlation of binding of a series of LEV family compounds to hSV2A assayed in transiently transfected COS-7 cells, pIC₅₀s measured against [3 H]ucb 30889, and of anti-seizure potencies in the mouse audiogenic model.

Figure 19 depicts the structure of ucb-101282-1. This ligand is a biotinylated derivative of ucb 30889.

Figure 20 depicts that the pKi of ucb-101282-1 is 6.3 (n=2) in rat brain membranes which is equivalent to the affinity reported for LEV.

Figure 21 (A, B, and C) depicts preparation of soluble SV2A and quantitation of by binding assay. A. Detection by western blot using anti-SV2A antibodies of soluble SV2A in the supernatant of solubilized rat brain membranes. B. Analysis of the ability of levetiracetam and ucb 30889 to specifically bind to soluble SV2A. C. Scatchard analysis indicates that the K_D for the binding of [3 H] ucb 30889 to SV2A in native rat brain membrane

is 30 nM, while that for the soluble protein is 82 nM.

Figure 22 depicts identification of SV2A partners. Western blot analysis show synaptotagmin associated to soluble SV2A in the immunopurified fractions of the supernatants from solubilized rat brain membranes. The isoform SV2B was not detected.

DETAILED DESCRIPTION

I. Synaptic Vesicle Protein 2 (SV2) Family of Proteins

Any SV2 protein that binds LEV or a derivative or analog thereof may be used in the assays herein described.

As used herein, SV2 proteins include isolated proteins, allelic variants of the proteins, and conservative amino acid substitutions of the proteins. As used herein, the "protein" or "polypeptide" refers, in part, to SV2A, a protein encoded by the nucleic acid sequence of SEQ ID NO: 1 or that has the human amino acid sequence depicted in SEQ ID NO: 2 or fragments thereof; to SV2B, which includes the human protein encoded by the nucleic acid sequence of SEQ ID NO: 3 or the amino acid sequence depicted in SEQ ID NO: 4 or fragments thereof; to SV2C, which includes the human protein encoded by the nucleic acid sequence of SEQ ID NO: 5 or the amino acid sequence depicted in SEQ ID NO: 6 or fragments thereof; and to SVOP, which includes the human protein encoded by the nucleic acid sequence of SEQ ID NO: 7 or the amino acid sequence depicted in SEQ ID NO: 8 or fragments thereof. The terms also refer to naturally occurring allelic variants and proteins that have a slightly different amino acid sequence than that specifically recited above. Allelic variants, though possessing a slightly different amino acid sequence than those recited above, will still have the same or similar biological functions associated with these proteins.

As used herein, the family of SV2 proteins related to the human amino acid sequence of SEQ ID NO: 2, 4, 6 or 8 refers in part, to proteins that have been isolated from organisms in addition to humans. For example, rat homologues of SV2A nucleic acid (SEQ ID NO: 9) and protein (SEQ ID NO: 10), SV2B nucleic acid (SEQ ID NO: 11) and protein (SEQ ID NO: 12), SV2C nucleic acid (SEQ ID NO: 13) and protein (SEQ ID NO: 14) and SVOP nucleic acid (SEQ ID NO: 15) and protein (SEQ ID NO: 16) have been identified and are included herein. The methods used to identify and isolate other members of the family of proteins related to these proteins are described below.

The SV2 proteins used in the present invention are preferably in isolated form in part of a cellular or vesicle membrane fragment, expressed in a transformed host cell, or naturally expressed in a given cell or tissue type. As used herein, a protein is said to be isolated when physical, mechanical or chemical methods are employed to remove the protein from cellular constituents that are normally associated with the protein. A skilled artisan can readily employ standard purification methods to obtain an isolated protein.

The SV2 proteins that may be used in the methods of the invention further include insertion, deletion, conservative amino acid substitution or splice variants of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16. As used herein, a "conservative" variant refers to alterations in the amino acid sequence that do not adversely affect the biological functions of the protein. A substitution, insertion or deletion is said to adversely affect the protein when the altered sequence prevents or disrupts a biological function associated with the protein. For example, the overall charge, structure or hydrophobic/hydrophilic properties of the protein can be altered without adversely affecting a biological activity. Accordingly, the amino acid sequence can be altered, for example to render the peptide more hydrophobic or hydrophilic, without adversely affecting the biological activities of the protein. As used herein, a "deletion" is defined as a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, are absent; an "insertion" or "addition" is that change in a nucleotide or amino acid sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring SV2 and a "substitution" results from the replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively.

SV2 proteins of the present invention further include fusion proteins, wherein a SV2 protein, or fragment thereof, is N- or C- terminally fused to another SV2 protein or fragment thereof, which may be the same as or different from the first SV2 protein or fragment thereof, and/or to a heterologous peptide fusion partner. The heterologous peptide may be a polypeptide sequence useful for the expression, purification, solubility, identification, antigenicity, or extension of the stability of the SV2 protein or fragment thereof. Heterologous fusion partners useful in the present invention include, but are not limited to, glutathione-S-transferase (GST), poly-histidine tags, green fluorescent protein (GFP), albumin, and ovalbumin or fragments thereof.

Ordinarily, the allelic variants, the conservative substitution variants, and the members of the SV2 protein family, will have an amino acid sequence having at least about 35%, 40%, 50%, 60%, 65%, 70% or 75% amino acid sequence identity with the full length sequence set forth in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16 more preferably at least about 80%, even more preferably at least about 90%, and most preferably at least about 95%, 97% or 99% sequence identity. Identity or homology with respect to such sequences is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the known peptides, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity (see section B for the relevant parameters). Fusion proteins, or N-terminal, C-terminal or internal extensions, deletions, or insertions into the peptide sequence shall not be construed as affecting homology.

Contemplated variants further include those containing predetermined mutations by, *e.g.*, homologous recombination, site-directed or PCR mutagenesis, and the corresponding proteins of other animal species, including but not limited to rabbit, mouse, rat, porcine, bovine, ovine, equine and non-human primate species, and the alleles or other naturally occurring variants of the family of proteins; and derivatives wherein the protein has been covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid (for example a detectable moiety such as an enzyme or radioisotope).

Fragments of the SV2 proteins may also be used in the methods of the invention. In particular, fragments comprising the LEV binding site may be used. Such fragments may have at least about 6 or 10, 15 or 20, or 25 or 30 amino acid residues, about 35 or 40 amino acid residues, about 45 or 50 amino acid residues, about 55 or 60, about 65 or 70 amino acid residues or at least about 75 or more amino acid residues

The methods of the present invention may also utilize nucleic acid molecules that encode members of the SV2 protein family, including, but not limited to, both the rat and human proteins known as SV2A, SV2B, SV2C and the related synaptic vesicle protein SVOP, such as those consisting of or comprising SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16 and the related proteins herein described, preferably in isolated form. Vectors, plasmids and transformed host cells may also be used to produce an SV2 protein. As used herein, "nucleic

acid" is defined as RNA or DNA or related molecules that encodes a protein or peptide as defined above, is complementary to a nucleic acid sequence encoding such peptides, hybridizes to such a nucleic acid and remains stably bound to it under appropriate stringency conditions, or encodes a polypeptide sharing at least about 35%, 40%, 50%, 60%, 65%, 70% or 75% sequence identity, preferably at least about 80%, more preferably at least about 85%, and even more preferably at least about 90%, 95%, 97% or 99% or more identity with the full-length peptide sequence of SEQ ID NO: 2, 4, 6, 8, or 10. The "nucleic acid molecules" useful in the invention further include nucleic acid molecules that share at least about 70% or 75% sequence identity, preferably at least about 80%, more preferably at least about 85%, and even more preferably at least about 90% and most preferably 95%, 97%, 99% or more identity with the nucleotide sequence of SEQ ID NO: 1, 3, 5, 7, or 9. Nucleic acids of the present invention also include those which encode fusion proteins comprising a SV2 protein either N- or C- terminally fused to a heterologous protein sequence or to another SV2 protein sequence.

Homology or identity at the nucleotide or amino acid sequence level is determined by **BLAST** (**B**asic **L**ocal **A**lignment **S**earch **T**ool) analysis using the algorithm employed by the programs **blastp**, **blastn**, **blastx**, **tblastn** and **tblastx** (Altschul, *et al.*, Nucleic Acids Res. 25: 3389-3402 (1997); Karlin *et al.*, Proc. Natl. Acad. Sci. USA 87:2264-2268 (1990)) which are tailored for sequence similarity searching. The approach used by the **BLAST** program is to first consider similar segments, with and without gaps, between a query sequence and a database sequence, then to evaluate the statistical significance of all matches that are identified and finally to summarize only those matches which satisfy a pre-selected threshold of significance. For a discussion of basic issues in similarity searching of sequence databases, see Altschul *et al.*, (Nature Genetics 6, 119-129 (1994)). The search parameters for **histogram**, **descriptions**, **alignments**, **expect** (*i.e.*, the statistical significance threshold for reporting matches against database sequences), **cutoff**, **matrix** and **filter** (low complexity) are at the default settings. The default scoring matrix used by **blastp**, **blastx**, **tblastn**, and **tblastx** is the **BLOSUM62** matrix (Henikoff *et al.*, Proc. Natl. Acad. Sci. USA 89:10915-10919 (1992)), recommended for query sequences over 85 in length (nucleotide bases or amino acids).

For **blastn**, the scoring matrix is set by the ratios of **M** (*i.e.*, the reward score for a pair

of matching residues) to **N** (*i.e.*, the penalty score for mismatching residues), wherein the default values for **M** and **N** are +5 and -4, respectively. Four **blastn** parameters were adjusted as follows: **Q**=10 (gap creation penalty); **R**=10 (gap extension penalty); **wink**=1 (generates word hits at every **wink**th position along the query); and **gapw**=16 (sets the window width within which gapped alignments are generated). The equivalent **Blastp** parameter settings were **Q**=9; **R**=2; **wink**=1; and **gapw**=32. A **Gap** comparison between sequences, available in the Accelrys' Wisconsin Package version 10.2, uses DNA parameters **GAP**=50 (gap creation penalty) and **LEN**=3 (gap extension penalty) and the equivalent settings in protein comparisons are **GAP**=8 and **LEN**=2.

"Stringent conditions" include those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS at 50°C, or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is hybridization in 50% formamide, 5× SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5× Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2× SSC and 0.1% SDS. A skilled artisan can readily determine and vary the stringency conditions appropriately to obtain a clear and detectable hybridization signal. Preferred molecules are those that hybridize under the above conditions to the complement of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13 or 15 and which encode a functional protein. Even more preferred hybridizing molecules are those that hybridize under the above conditions to the complement strand of the open reading frame of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13 or 15.

As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid molecule is substantially separated from contaminant nucleic acid molecules encoding other polypeptides.

A. SV2A and the Levetiracetam Binding Site (LBS)

The invention includes the characterization and use of the LBS located on the SV2A protein.

As described above, "SV2A" includes the human protein as described in SEQ ID NO: 2, the human protein encoded by SEQ ID NO: 1, species homologues of human SV2A, variants of SEQ ID NO: 2 as herein described, and fragments of SV2A comprising the LBS.

5 II. Levetiracetam and Analogs

The methods of the invention include the use of LEV and LEV analogs or derivatives thereof in assays to identify new pharmacological agents. In a preferred embodiment, the methods of the present invention identify compounds or agents that compete with LEV and LEV analogs or derivatives thereof for binding to the LBS of SV2. As used herein, the terms
10 "compete" and "competitive binding" refer to agents or compounds which occupy the same binding site on the LBS as LEV or analogs or derivatives thereof; displace, or are displaced by, LEV or analogs or derivatives thereof in binding to the LBS; or inhibit, or are inhibited by, LEV or analogs or derivatives thereof in binding to the LBS. In another preferred embodiment, the invention includes the identification of compounds or agents that modulate
15 the activity of SV2A. In another preferred embodiment the methods of the present invention identify compounds or agents which have less, about the same, or greater affinity for the LBS than LEV. In yet another preferred embodiment the methods of the present invention identify compounds or agents which have less, about the same, or greater affinity for the LBS than ucb 30889. In still another preferred embodiment the methods of the present invention
20 identify compounds or agents which in an effective amount modulate the activity of SV2A for a longer period of time than an effective amount of LEV. In even another preferred embodiment the methods of the present invention identify compounds or agents which in an effective amount modulate the activity of SV2A for a shorter period of time than an effective amount of LEV.

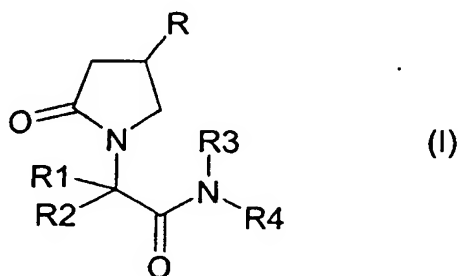
25 As used herein, "levetiracetam" (Figure 15A; LEV), refers to the International Non-proprietary name of the compound (S)- α -ethyl-2-oxo-1-pyrrolidine acetamide as disclosed in European Patent No. 0 162 036 B1, herein incorporated by reference in its entirety. LEV is a levorotary compound which is a protective agent for the treatment and prevention of hypoxic and ischemic type aggressions of the central nervous system. This compound is also
30 effective in the treatment of epilepsy. Racemic α -ethyl-2-oxo-1-pyrrolidine acetamide and

analogues thereof are known from British Patent No. 1 309 692. US Patent No. 3,459,738 discloses derivatives of 2-oxo-1-pyrrolidine acetamide.

As used herein, the term "LEV analogs or derivatives thereof" includes optionally substituted N-alkylated 2-oxo-pyrrolidine derivatives. Preferably, those compounds are alkyl amides derivatives substituted on the positions 4 and/or 5 of the pyrrolidone ring. Examples of optionally substituted N-alkylated 2-oxo-pyrrolidine derivatives include, but are not limited to, compounds such as those disclosed in international patent application PCT/EP01/01992 such as (2S)-2-[(4S)-4-(2,2-difluorovinyl)-2-oxopyrrolidinyl]butanamide, (2S)-2-[(4R)-2-oxo-4-propylpyrrolidinyl]butanamide, (2S)-2-[(4S)-2-oxo-4-propylpyrrolidinyl]butanamide, and (2S)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide.

As used herein, the term "LEV analogs or derivatives thereof" further include optionally substituted N-alkylated 2-oxo-piperidinyl derivatives. Preferably, those compounds are alkyl amides derivatives substituted on the position 4 and/or 5 and/or 6 of the 2-oxo-piperidinyl ring. Examples of optionally substituted N-alkylated 2-oxo-pyrrolidine derivatives include, but are not limited to, compounds such as those disclosed in international patent application PCT/EP02/05503 such as (2S)-2-[5-(iodomethyl)-2-oxo-1-piperidinyl]butanamide, (2S)-2-[5-(azidomethyl)-2-oxo-1-piperidinyl]butanamide, 2-(2-oxo-5-phenyl-1-piperidinyl]butanamide, (2S)-2-[4-(iodomethyl)-2-oxo-1-piperidinyl]butanamide, and (2S)-2-[4-(2-fluoro-2-methylpropyl)-2-oxo-1-pyrrolidinyl]butanamide.

As used herein, the term "LEV analogs or derivatives thereof" includes any acetam compound of formula I, in racemic or isomeric form, or a pharmaceutically acceptable salts thereof,



- R represents hydrogen or hydroxy;
- R¹ and R² represent independently hydrogen or an alkyl group of 1-4 carbon atoms; and
- R³ and R⁴ represent independently hydrogen, an alkyl group of 1-4 carbon atoms or $-(CH_2)_n - NR^5R^6$ wherein n is 1, 2 or 3 and R⁵ and R⁶ represent independently hydrogen or an alkyl group of 1-4 carbon atoms.

An example of such an acetam compound includes, but is not limited to, a compound of formula I wherein R, R¹, R², R³ and R⁴ are hydrogen, 2-oxo-pyrrolidineacetamide, known by the generic name piracetam as described in UK Patents Nos. 1,039,113 and 1,309,692.

As used herein, the term "LEV analogs or derivatives thereof" also include optionally substituted N-alkylated 2-oxo-azepanyl derivatives. Preferably, those compounds are alkyl amides derivatives substituted on the positions 4 and/or 5 and/or 6 and/or 7 of the 2-oxo-azepanyl ring. Examples of optionally substituted N-alkylated 2-oxo-azepanyl derivatives include, but are not limited to, compounds such as those disclosed in international patent application PCT/EP02/05503 such as 2-[5-(iodomethyl)-2-oxo-1-azepanyl]butanamide.

In another embodiment the present invention includes compounds or agents which are derivatives or analogs of piracetam which bind to the LBS. Such compounds would also include molecules such as aniracetam and nefiracetam. In a preferred embodiment, the derivatives or analogs of piracetam are those which modulate the activity of SV2A or other SV2 family members.

III. Assay Formats

Assays of the present invention include methods of identifying agents or compounds which are useful for the treatment of neurological disorders, such as seizures, epilepsy, Parkinson's disease, Parkinson's dyskinesias, migraine, Alzheimer's disease, neuropathic pain, essential tremor, cognitive disorders, movement disorders, endocrinopathy and adrenal-medulla-related disease, such as hypoglycemia and circulation shock. Assays of the present invention also include methods of identifying agents or compounds which have cognitive enhancing effects, such as for example might be measured in animal models of cognition. In particular, the assays of the present invention include methods of identifying agents or

compounds that compete with LEV or analogs or derivatives thereof for binding to the LBS of SV2A, displace, or are displaced by, LEV or analogs or derivatives thereof in binding to the LBS; or inhibit, or are inhibited by, LEV or analogs or derivatives thereof in binding to the LBS.

5 LEV, ucb 30889 (Figure 15B) and other derivatives or analogs of LEV as described above are useful in the methods of the invention as binders in assays to screen for new compounds or agents that bind to the LBS of SV2A. In such assay embodiments, LEV, ucb 30889 and derivatives or analogs can be used without modification or can be modified in a variety of ways; for example, by labeling, such as covalently or non-covalently joining a moiety which directly or indirectly provides a detectable signal. In any of these assays, the materials can be labeled either directly or indirectly. Possibilities for direct labeling include label groups such as: radiolabels including, but not limited to, [^3H], [^{14}C], [^{32}P], [^{35}S] or [^{125}I], enzymes such as peroxidase and alkaline phosphatase, and fluorescent labels capable of monitoring the change in fluorescence intensity, wavelength shift, or fluorescence polarization, including, but not limited to, fluorescein or rhodamine. In addition, FRET techniques could be used to analyze interactions between ligands and the LBS of SV2A. Possibilities for indirect labeling include biotinylation of one constituent followed by binding to avidin coupled to one of the above label groups or the use of anti-ligand antibodies. The compounds may also include spacers or linkers in cases where the compounds are to be attached to a solid support.

To identify agents or compounds which compete or interact with LEV and ucb 30889 and derivatives for binding to the LBS of SV2A, intact cells, cellular or membrane fragments containing SV2A or the entire SV2A protein or a fragment comprising the LBS of the SV2A protein can be used. The agent or compound may be incubated with the cells, membranes, SV2 protein or fragment prior to, at the same time as, or after incubation with LEV or an analog or derivative thereof. Assays of the present invention can measure any property or function known for SV2 proteins, synaptic vesicles, neural transmission and/or endocrine cell function, as well as presynaptic accumulation of divalent cations, including Ca^{2+} . Examples of properties or functions of an SV2 protein which may be measured as an assay endpoint include, but are not limited to, phosphorylation state, binding of divalent cations, including Ca^{2+} ; membrane transport; transport of divalent cations (including Ca^{2+}) into and/or out of

synaptic vesicles; transport of neurotransmitters (including, but not limited to amines, acetylcholine, excitatory neurotransmitters, GABA, serotonin, and glycine) into and/or out of synaptic vesicles; interaction with other proteins (including, but not limited to laminins and synaptotagmin); conformational changes, as measured by sensitivity to proteolysis or other
5 changes in biochemical or biophysical properties; divalent cation channel formation; formation or dissociation of protein complexes; synaptic vesicle function; fusion; exocytosis; and synaptic vesicle recycling.

Assays of the invention may be modified or prepared in any available format, including high-throughput assays that monitor the binding of LEV or the binding of
10 derivatives or analogs thereof to SV2A or to the LBS of the SV2A protein. In many drug screening programs which test libraries of compounds, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Such screening assays may use intact cells, cellular or membrane fragments containing SV2A as well as cell-free or membrane-free systems, such as may be derived with purified or
15 semi-purified proteins. The advantage of the assay with membrane fragment containing SV2A or purified SV2A proteins and peptides is that the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an inhibition of, for instance, binding between two molecules.

20 In one embodiment of a competitive screening assay, the assay can be formulated to detect the ability of a test agent or compound to inhibit binding of ucb 30889 to SV2A or a fragment of SV2A comprising the LBS or of LEV, or derivatives or analogs thereof, to SV2A or a fragment of SV2A comprising the LBS. In another embodiment of a competitive screening assay, the assay can be formulated to detect the ability of ucb 30889 or of LEV, or
25 derivatives or analogs thereof, to inhibit binding of a test agent or compound to SV2A or a fragment of SV2A comprising the LBS. The inhibition of complex formation may be detected by a variety of techniques. For instance, modulation of the formation of complexes can be quantitated using, for example, detectably labeled ucb 30889, LEV, or derivatives or analogs of LEV. The inhibition of complex formation may be detected by using a detectably
30 labeled version of the agent or compound being assayed for competitive binding to the LBS of SV2A. Alternatively, the binding between the SV2A protein and a ligand may be detected

with no need of a labeled probe. For instance surface plasmon resonance, nuclear magnetic resonance or mass spectrometry are the instruments of choice for such binding assays. Another method is to measure changes in the sensitivity of SV2 proteins to proteases induced by binding of a ligand.

5 In certain instances, it will be desirable to immobilize one of the LBS (SV2A or a fragment of SV2A comprising the LBS) or the ligand (LEV, ucb 30889 or the test agent or compound) to facilitate separation of complexes from uncomplexed forms, as well as to accommodate automation of the assay. Binding of a ligand to the LBS, for instance binding of a candidate agent or compound to SV2A, in the presence and absence of LEV or ucb
10 30889, can be accomplished in any vessel suitable for containing the reactants. Examples include microtiter plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows the LBS to be bound to a matrix. For example, glutathione-S-transferase(GST) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized
15 microtiter plates, which are then combined with the labeled LEV, ucb 30889, or derivatives or analogs of LEV and the unlabeled test agent or compound; or alternatively, with the unlabeled LEV, ucb 30889, or derivatives or analogs of LEV and the labeled test agent or compound. The mixture is then incubated under conditions conducive to complex formation. Following incubation, the beads are washed to remove any unbound reactants, and the matrix
20 immobilized label determined directly, or in the supernatant after the LBS/ligand complexes are subsequently dissociated. When amenable, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of ligand found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

Other techniques for immobilizing proteins on matrices are also available for use in
25 the subject assay. For instance, the LBS can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, Ill.), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with the LBS but which do not interfere with
30 ligand binding can be derivatized to the wells of the plate, and LBS binding trapped in the wells by antibody conjugation. As above, preparations of a ligand and a test compound are

incubated in the protein-presenting wells of the plate, and the amount of protein/ligand complex trapped in the well can be quantitated. Exemplary methods for detecting such complexes, in addition to those described above, include immunodetection of complexes using antibodies reactive with the ligand, or which are reactive with the protein and compete
5 for binding with the ligand.

In another embodiment of the invention, competitive binding assays can be carried out using cellular extracts of cells or tissues that comprise the LBS to identify SV2 binding partners. As used herein, a cellular extract refers to a preparation or fraction that is made from a lysed or disrupted cell. The preferred source of cellular extracts will be cells derived
10 from human central nervous tissue or endocrine tissues. In particular, cellular extracts may be prepared from a particular region, including, but not limited to, the hippocampus, the cerebellum, the cerebrum, the cerebral cortex, the pituitary, the medulla, and the adrenal gland. Further, cellular extracts may be prepared from a particular primary cell isolate of central nervous system origin or the endocrine systems including, but not limited to, neurons,
15 astrocytes, and endocrine cells of the medulla. Alternatively, cellular extracts may be prepared from available cell lines, particularly cell lines of a neurological or endocrine origin. Cell lines contemplated herein include, but are not limited to, rat PC12 pheochromocytoma cells, AtT-20, GH3 and HIT cells.

A variety of methods can be used to obtain an extract of a cell. Cells can be disrupted
20 using either physical or chemical disruption methods. Examples of physical disruption methods include, but are not limited to, sonication and mechanical shearing. Examples of chemical lysis methods include, but are not limited to, detergent lysis and enzyme lysis. A skilled artisan can readily adapt methods for preparing cellular extracts in order to obtain extracts for use in the present methods.

25 Once an extract of a cell is prepared, the extract is mixed with SV2 protein or fragment and other components of the assay under conditions in which association of the protein with the binding partner can occur, followed by the addition of LEV or an analog or derivative thereof. Alternatively, the LEV or an analog or derivative thereof may be added to the cellular extract before or at even time with the test agent or compound. A variety of
30 conditions can be used, the most preferred being conditions that closely resemble conditions found in the cytoplasm of a human cell. Features such as osmolarity, pH, temperature, and

the concentration of cellular extract used, can be varied to optimize the association of the protein with the binding partner.

After mixing under appropriate conditions, the bound complex is separated from the mixture. A variety of techniques can be utilized to separate the mixture. For example, antibodies specific to SV2A can be used to immunoprecipitate the binding partner complex. Alternatively, standard chemical separation techniques such as chromatography and density/sediment centrifugation can be used.

After removal of non-associated cellular constituents found in the extract, the binding partner can be dissociated from the complex using conventional methods. For example, dissociation can be accomplished by altering the salt concentration or pH of the mixture.

As discussed above, to aid in separating associated binding partner pairs from the mixed extract, the LBS can be immobilized on a solid support. For example, the LBS can be attached to a nitrocellulose matrix or acrylic beads. Attachment of the LBS to a solid support aids in separating peptide/binding partner pairs from other constituents found in the extract. The identified binding partners can be either a single protein or a complex made up of two or more proteins. Alternatively, binding partners may be identified using a Far-Western assay according to the procedures of Takayama *et al.* (Methods Mol. Biol. 69:171-184. (1997)) or Sauder *et al.* (J. Gen. Virol. 77:991-996. (1996)) or identified through the use of epitope tagged proteins or poly-His fusion or GST fusion proteins.

Alternatively, mammalian cell-based protein-protein assays utilizing bioluminescence or fluorescence energy transfer (BRET and FRET, respectively) and the yeast two-hybrid system may be a tool for the identification of protein-protein interactions.

Another approach to identifying pharmacologically active compounds that act via the SV2 proteins is by analyzing the effects of such compounds on wild-type and SV2 knockout cell lines, tissues, and animals. For example, compounds of interest, which might have previously been identified by testing in genomic wild-type animal or tissue models of disease, or by screening against functional cellular assays, can be re-tested in equivalent or informative assays in cells, tissues or animals that have reduced or low levels of functional SV2 proteins, or which lack functional SV2 proteins altogether. Such knockdowns or knockouts might be obtained, for example, by using anti-sense or RNAi techniques, or by working with genomic knockout animals.

In some embodiments, compounds that inhibit N-type calcium channels in neurons of wild-type animals are identified, followed by testing the compounds under the same conditions in neurons that have their SV2 proteins knocked down using RNAi or antisense oligos targeted to the SV2 mRNA sequences, or, alternately, neurons from genomic SV2 knockout animals. The lack of an effect in the SV2 knockout neurons would be evidence that the compounds are having their effect via SV2 proteins.

In another embodiment, compounds with anticonvulsant properties are identified by testing their ability to inhibit epileptiform field potentials recorded in the CA3 area of wild-type rat hippocampal slices bathed in an epileptogenic medium containing increased potassium and lowered calcium. Compounds that exhibit anticonvulsant properties could then be tested in the same assay using SV2 knockout or knockdown hippocampal slices. If a lack of efficacy was observed in the slices without SV2 protein expression, this would strongly support an effect mediated by interactions with SV2 proteins.

In another embodiment, the effect of compounds or agents which bind to the LBS on presynaptic divalent cation storage can be studied in knockout or knockdown mice. In a particular embodiment, wild-type and SV2 knockout or knockdown mice are administered an amount of the compound or agent which binds to the LBS. Animals are sacrificed and brains are immediately removed and flash-frozen. Elemental imaging of thin freeze-dried cryosections is carried out and the elemental composition of the presynaptic nerve terminals is determined by electron probe x-ray microanalysis and elemental imaging of characteristic x-rays. An example of such a method is disclosed by Andrews *et al.* (Proc. Natl. Acad. Sci. USA 84(6):1713-1717 (1987)).

IV. *In Vitro* Characterization of SV2

The invention includes the functional characterization of the SV2 family of proteins. In one embodiment, the invention includes the cloning and expression of both the rat and human forms of the SV2 proteins SV2A, SV2B, SV2C and the related synaptic vesicle protein SVOP. In another embodiment, the invention further includes identification of the domain or domains comprising the LBS. In an additional embodiment, the invention includes discovery of possible multiple functions of the SV2 proteins, and of the effect(s) of levetiracetam and related ligands on these functions.

In an additional embodiment, the invention includes expression of the SV2 protein in a eukaryotic host cell for study of function. The protein might be expressed in its native form, or as fusions with fluorescent or other peptidic tags, including epitope and affinity tags; mutant forms, or fragments of the protein might be expressed and studied, fusions between
5 the protein and homologous proteins might be expressed and studied. The heterologously expressed SV2 might be studied in-situ using electrophysiology, microscopy, or other techniques; or it might be expressed and purified in functional form from the eukaryotic using electrophysiology or other techniques.

In one particular embodiment, the SV2 protein, native or modified as above, might be
10 expressed in a eukaryotic host and purified. The protein might be purified and incorporated into artificial lipid vesicles, or artificial bilayer membranes for study. Possible transport functions of the SV2 protein might be studied by biochemical means, for instance measuring radioactively labeled substrate transport into or out of the vesicles. Another possible approach is to use electrophysiology to study such transport in purified protein incorporated
15 into synthetic vesicles or artificial lipid membranes.

In another particular embodiment, the invention includes the expression of the SV2 protein in a prokaryotic host, such as *E. coli*, and purification. In another particular embodiment, the invention includes the recombinant expression of the SV2 protein in a eukaryotic host, including yeast (*Saccharomyces cerevisiae* or *Pichia pastoris*, for example),
20 COS-7, HEK293 and PC12a cells, and purification. In accordance with the present invention, polynucleotide sequences that encode SV2 proteins, fragments of the polypeptide, fusion proteins or functional equivalents thereof may be used to generate recombinant DNA molecules that direct the expression of SV2 protein in appropriate host cells. Due to the inherent degeneracy of the genetic code, other DNA sequences that encode substantially the
25 same or a functionally equivalent amino acid sequence may be used to clone and express SV2. As will be understood by those of skill in the art, it may be advantageous to produce SV2-encoding nucleotide sequences possessing non-naturally occurring codons. Codons preferred by a particular prokaryotic or eukaryotic host (Murray *et al.* Nuc. Acids Res. 17:477-508. (1989)) can be selected, for example, to increase the rate of SV2 expression or to
30 produce recombinant RNA transcripts having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

In another embodiment, SV2 proteins of the invention are recombinantly co-expressed in host cells with other proteins SV2 is normally associated with in synaptic vesicles. In a preferred embodiment, SV2 proteins are co-expressed with SNARE complex proteins including vesicle-associated VAMP/synaptobrevin, syntaxin and SNAP-25. In a preferred embodiment, SV2A is recombinantly co-expressed in a host cell with recombinantly expressed synaptotagmin.

In another embodiment, the roles of glycosylation, phosphorylation and other natural or introduced protein modifications in SV2 protein function, stability and interaction are analyzed. Nucleotide sequences encoding SV2 proteins of the present invention can be engineered using methods generally known in the art in order to alter sequences encoding SV2 proteins for a variety of reasons, including but not limited to; alterations which modify the cloning, processing, and/or expression of the gene product; alterations which modify the interaction of SV2 proteins with binding partners; alterations of the solubility and/or membrane insertion of SV2 proteins; and alterations which affect the LBS and its association with ligands. DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides may be used to engineer the nucleotide sequences. For example, site-directed mutagenesis may be used to insert new restriction sites, to alter glycosylation patterns, to change codon preference, to produce splice variants, or to introduce mutations, and so forth.

Upon exocytosis at the synapse, vesicles cluster at the pre-synaptic plasma membrane and fuse in response to increased Ca^{2+} levels. Upon Ca^{2+} accumulation within the synapse, the binding of synaptotagmin to SV2A is inhibited and dimerization of two synaptotagmin Ca^{2+} -binding domains (C2B) is stimulated, which may play a role in organizing the SNARE complex and promoting fusion. At low Ca^{2+} , the fusion of vesicles is inhibited because SV2A is still attached to the synaptotagmin complex. Binding of synaptotagmin to other proteins, including the ATPase VCP, the SNARE protein SNAP-25 and syntaxin, is Ca^{2+} -dependent (Augustine, 2001). In order to shed light on this exocytosis mechanism and define more precisely the role of SV2A in the fusion process, changes in protein levels within these complexes in response to modulation of the LBS are assayed. In particular embodiments, the ability of LBS ligands to modulate the interactions between SV2A and the synaptotagmin-SNARE complex and to assess which stage of the complex assembly and which partners are

modulated by the binding to SV2A of LEV, analogs or derivatives thereof, or compounds or agents which compete with LEV for binding to the LBS. In one such embodiment, protein stoichiometry in the complex after ligand addition is analyzed using antibodies specific for the identified SV2A partners and a combination of immunoprecipitation and recombinant
5 GST-fusion protein affinity chromatography.

In another such embodiment, mass spectrometry and/or surface plasmon resonance are used to detect the effects of LBS ligands on the interactions between SV2A and its partner (e.g. synaptotagmin) or short peptides derived from binding domains. In another particular embodiment, biochemical approaches are used to demonstrate if LBS ligands compete with
10 bivalents (such as Ca^{2+} , Pb^{2+} , Zn^{2+}) and inhibit their interactions with SV2A and/or synaptotagmin. In another particular embodiment, the role of the SV2 proteins in synaptic vesicle fusion and recycling is analyzed by the creation of PC12a cell lines, primary neuronal cultures, chromaffin cells and other cell lines or primary isolates expressing fusion constructs between the SV2 proteins and GFP. In one such embodiment, these cell lines are analyzed
15 by fluorescence microscopy tracking of SV2 complexes and synaptic vesicle exocytosis and trafficking, and the effects of treatments with LBS ligands on these events. The cell types described above can also be used to measure vesicle fusion and exocytosis (using encapsulated dye into the vesicles or measuring the release of labeled neurotransmitters) and the ability of LBS ligands to modulate these activities.

20 In other embodiments of characterizing SV2 proteins and its binding partners, enrichment of the entire multi-protein complex is achieved by affinity-based methods using GST-fusion SV2 or anti-SV2 antibodies. In a particular embodiment, SV2A is overexpressed in PC12 cells with a GST tag and, together with its partners, immunoprecipitated by an antibody against the tag. In a related embodiment, SV2A is immobilized onto agarose beads
25 using a GST or poly-histidine tag. In a preferred embodiment, synaptic vesicle extracts, cell extracts or brain extracts are incubated with the beads, SV2A is cleaved off and eluted proteins are resolved by 1D or 2D gels and analyzed. In a further embodiment, identification of these proteins is used to search databases for novel putative interacting partners. In another embodiment, the yeast two-hybrid (Y2H) system or mammalian cell-based protein-
30 protein assays are used for the identification of protein-protein interactions within living organisms to confirm SV2 binding partners found by the affinity-based methods and to define

the specific protein domain interactions using known cDNAs.

Antibodies specific for SV2 proteins may be produced by inoculation of an appropriate animal with the polypeptide or an antigenic fragment. Antibodies specific for the LBS may be produced by inoculation with full-length SV2 protein or a fragment comprising the LBS. An antibody is specific for the particular SV2 if it is produced against an epitope of the polypeptide and binds to at least part of the natural or recombinant protein. Monoclonal and/or polyclonal antibodies specific for SV2 or for the LBS may be produced by any of a number of methods which are well known in the art for antibody production, such as those taught by Harlow and Lane (Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. (1988)). SV2 peptides for antibody induction do not require biological activity; however peptides must be immunogenic. Peptides used to induce specific antibodies may have an amino acid sequence consisting of at least five amino acids, preferably at least 10 amino acids. They should mimic a portion of the amino acid sequence of the natural protein and may contain the entire amino acid sequence of a small, naturally occurring molecule. Short stretches of SV2 amino acids may be fused with those of another protein such as keyhole limpet hemocyanin and antibody produced against the chimeric molecule. Antibody production includes not only the stimulation of an immune response by injection into animals, but also analogous steps in the production of synthetic antibodies or other specific-binding molecules such as the screening of recombinant immunoglobulin libraries (see *e.g.* Orlandi *et al.* Proc. Nat. Acad. Sci. USA 86:3833-3837. (1989); Huse *et al.* Science 256:1275-1281. (1989)) or the *in vitro* stimulation of lymphocyte populations. Current technology (Winter G. and Milstein C. (1991) Nature 349:293-299) provides for a number of highly specific binding reagents based on the principles of antibody formation. These techniques may be adapted to produce molecules specifically binding SV2 or the LBS.

In a particular embodiment, the present invention includes the human SV2C protein of SEQ ID NO: 6 and the nucleic acid molecule encoding it (SEQ ID NO: 5), as well as allelic variants and functional equivalents thereof. The invention further includes identification of the *in vivo* distribution of the SV2C protein, including, but not limited to, within the central nervous system, peripheral nervous system and endocrine cells and tissues. The invention further includes identification of ligands and/or binding partners of the SV2C

protein. The invention further includes elucidation of the function of the SV2C protein.

V. SV2 Expression in Disease

The invention includes elucidating the expression of SV2 proteins in relation to
5 specific neurological diseases. In a particular embodiment, antibodies specific for SV2A are
used to probe brain tissue in a regional specific manner within the brain, spinal cord and
neuroendocrine tissues or cells such as chromaffin cells of control animals and animals
mimicking epilepsy, epileptogenesis, Parkinson's disease and cognition deficits, other CNS
disorders (see above) and endocrinopathy and adrenal medulla-related diseases. In another
10 embodiment, the invention includes the elucidation of the relationship of all SV2 protein
isoforms to the pathologies described above, including alterations or switching of isoforms.
In a preferred embodiment DNA microarrays are probed for the expression of SV2 protein
coding sequences, and changes thereof, in relation to different neurological diseases. An
example of using DNA microarrays for determining expression of a particular nucleic acid
15 sequence can be found in U.S. Patent 5,900,882. In another preferred embodiment, changes
in regional or global SV2 protein expression in relation to a neurological disorder associated
with synaptic vesicle function is validated by quantitative PCR (qPCR).

In another embodiment, knockout mice are analyzed for the presence of the LBS. In a
preferred embodiment, purified synaptic vesicles from mice with the lethal SV2A knockout
20 phenotype, SV2B or double KO SV2A/B are purified and analyzed for the presence of the
LBS and for substrate and/or ion uptake in comparison with synaptic vesicles from wild-type
mice.

In a particular embodiment, comparisons are made of protein expression levels in
synaptic vesicles purified from healthy and diseased animals including, but not limited to,
25 pathologies described above, for example, for protein mapping of synaptic vesicles for the
detection of disease-related proteins. In a particular embodiment, comparison of 1D and/or
2D gels of synaptic vesicles derived from the healthy and the diseased states are used to
identify proteins that are up- or down-regulated in a disease-specific manner. In another
embodiment, targets are identified by comparison of the proteome of synaptic vesicles from
30 wild-type with that of SV2 knock-out mice or double-stranded RNA-induced interference
(RNAi; Krichevsky *et al.*, Proc. Nat. Acad. Sci. USA 99(18):11926-11929. (2002)) cultured

neurons. In another embodiment, the invention includes performing RNAi or antisense nucleotides in primary neuronal cultures, cultured neurons or PC12 cells to inhibit or eliminate SV2 expression.

5 VI. Determination of the LBS Location on the SV2 Protein

A number of methods are employed in the determination of the location of the LBS. The LBS may be comprised of a contiguous segment of amino acid residues, or it may be a 3 dimensional structure comprised of amino acid sequences present on one or more extracellular or intracellular loops or domains. In addition, the LBS may be dependent upon
10 the glycosylation of the SV2 protein or may not require glycosylation of the SV2 protein.

In a particular embodiment, radioligands are used to specifically photoaffinity-label the LBS. In a particular embodiment, the site of covalent attachment of the radioligand is determined by purifying and sequencing the proteolytic fragment from photoaffinity labeled synaptic vesicles with SV2A-antibody affinity chromatography or immunoprecipitation and
15 mass spectrometry.

In particular embodiments for the identification of protein domains involved in the interactions between LBS ligands and the SV2A protein fragments of SV2 proteins, or SV2 proteins with amino acid deletions, additions or substitutions are analyzed for effects on binding. In a preferred embodiment, selected residues will be modified by site directed
20 mutagenesis of the cDNA. In another embodiment, domains are exchanged between SV2 isoforms and structural features of isoforms that are important for ligand recognition are identified. In an example of this embodiment, the N-terminal domain of SV2A is replaced with the shorter equivalent region of SV2B to determine the effect on LBS ligand binding. In another example of this embodiment, a series of swaps are made between regions of SV2A
25 and regions of SVOP, to determine the effect on ligand binding. Such swaps might include large regions of each protein, containing for example, multiple transmembrane regions, as well as small regions of the protein, including for example individual transmembrane regions.

In another embodiment, the three-dimensional structure of the SV2 protein (or selected binding domains) is analyzed using NMR spectroscopy or x-ray crystallography or
30 circular dichroism or infrared spectroscopy utilizing pure SV2A with at least binding activity maintained for the revelation of resolution of the topology of LBS sites and design of new

drugs to fit that receptor. If the binding domain upon investigation requires an hydrophobic environment then the protein must be solubilized in a detergent such as dodecylmaltoside or derivatives (see Examples). Purified protein can be crystallized by methods known in the art, for example, by methods disclosed by A. McPherson in "Preparation and Analysis of Protein Crystals" (John Wiley and Sons, New York, (1982)). Alternatively, SV2 proteins of the present invention may also be crystallized by vapor diffusion and vapor diffusion apparatus used in the art may be readily employed in the processes of the present invention. Such apparatus are disclosed in, for example, U.S. Pat. Nos. 4,886,646; 5,096,676; 5,130,105; 5,221,410 and 5,400,741, the disclosure of which are herein incorporated by reference. X-ray crystallography determination of SV2 protein structure as well as its association with ligands and/or binding partners can be performed using methods and imaging systems as disclosed in U.S. Patent No. 5,978,444, for example.

In some embodiments, SV2 proteins, including isoforms SV2A, SV2B and SV2C, are recombinantly expressed in host cell lines to screen a diverse set of compounds or agents in binding assays for each isoform. Compounds or agents that interact with the SV2A isoform are analyzed for interaction with other SV2 isoforms. In another embodiment, binding experiments are performed to test several reference drugs, AEDs, steroids and to compare the kinetics of binding between native LBS, human and rat recombinant SV2A.

VII. Uses for Agents on the Invention

The invention includes the use of the compounds or agents identified by methods of the invention for the modulation of SV2 protein. Compounds or agents of the invention can be used to modulate synaptic vesicle function; in particular to modulate disorders associated with synaptic vesicle function, or disorders which might be improved by affecting some aspect of synaptic vesicle function, or also to modulate synaptic vesicle function in order to correct disorders of pre-synaptic function, or disorders of neuronal signaling that can be fixed by compensatory changes in synaptic vesicle function. As used herein, a compound or agent is said to modulate synaptic vesicle function if it is capable of up- or down-regulating at least one function of at least one component of a synaptic vesicle, or the pre-synaptic systems which synaptic vesicles interact with.

In a preferred embodiment, the agent or compound is LEV or an analog or derivative

thereof. In another preferred embodiment, the compound or agent binds to the levetiracetam binding site of an SV2 protein. In still another preferred embodiment, the compound or agent competes with levetiracetam or an analog or derivative thereof for binding to the levetiracetam binding site. In yet another preferred embodiment, agents of the invention for the treatment of neurological disorders include N-alkylated 2-oxo-pyrrolidine derivatives, N-alkylated 2-oxo-piperidinyl derivatives, and N-alkylated 2-oxo-azepanyl derivatives as described above.

In a preferred embodiment, the compound or agent is an anti-SV2 antibody or fragment thereof, including those that bind to the levetiracetam binding site of SV2 protein and may be a polyclonal antibody or a monoclonal antibody. In related preferred embodiments, the antibody fragment is an Fab fragment, Fab' fragment, F(ab')₂ fragment or an scFv fragment, whereas the monoclonal antibody is a chimeric antibody, humanized antibody, or a human antibody.

In a preferred embodiment, the invention includes the modulation at least one function or activity of a SV2 protein in a cell, for example, by exposing the cell to a compound or agent that binds to the levetiracetam binding site of the SV2 protein. In particular embodiments, modulation at least one function or activity of a SV2 protein in a cell includes exposure of the cell to the compound or agent *in vitro*, *in vivo*, *in situ* and *ex vivo*. As used herein, modulation of a function of SV2 includes, but is not limited to modulation of the transport of ions or other natural substrates across the membrane of the synaptic vesicle, modulation of the binding of an SV2 protein to a natural ligand thereof, modulation of the binding of an SV2 protein to a binding partner as described above, and remodeling of synaptic vesicle formation, fusion, regulation or function.

In a preferred embodiment, the modulation of SV2 protein in a cell includes modulating synaptic vesicle function in the cell. As used herein, synaptic vesicle functions which may be modulated by compounds or agents identified by the methods of the invention include, but are not limited to, formation of synaptic vesicles in the presynaptic neuron, fusion of synaptic vesicles with other synaptic vesicles or the synaptic membrane, recycling or turnover of synaptic vesicles, association of synaptic vesicles with the presynaptic grid, and neurotransmitter release, association with proteins from the extracellular matrix (laminin-1, etc.) and post-synaptic densities.

In particular embodiments, exposure of the cell to a compound or agent of the invention which modulates at least one function or activity of a SV2 protein in a cell is carried out under conditions where the concentration of monovalent and/or divalent cations in the environment of the cell is controlled. In preferred embodiments, the divalent cation is at least one of Ca^{2+} , Zn^{2+} , Pb^{2+} , Mg^{2+} , Mn^{2+} , Fe^{2+} and Cu^{2+} . In preferred embodiments, the monovalent cation is K^{+} . In a particular embodiment, exposing the cell to a compound or agent which binds to the levetiracetam binding site is carried out under conditions with a low monovalent and/or divalent cation concentration, or less than about $1\ \mu\text{M}$. In another particular embodiment, exposing the cell to a compound or agent which binds to the levetiracetam binding site is carried out under conditions with a physiological monovalent and/or divalent cation concentration, or between about $1\ \mu\text{M}$ and about $1000\ \mu\text{M}$. In yet another particular embodiment, exposing the cell to a compound or agent which binds to the levetiracetam binding site is carried out under conditions with a high monovalent and/or divalent cation concentration, or more than at least about $1000\ \mu\text{M}$.

VIII. Treatment Of Neurological Disorders

Compounds or agents identified by the methods of the invention can be used in an effective amount to treat neurological disorders associated with synaptic vesicle function. In a particular embodiment, treatment with the compound or agent modulates a neurological disorder. In a preferred embodiment, the neurological disorder is a seizure disorder. In another preferred embodiment, the neurological disorder is selected from the group consisting of Parkinson's disease, Parkinson's dyskinesias, migraine, Alzheimer's disease, neuropathic pain, essential tremor, and cognitive disorders. In a highly preferred embodiment, the neurological disorder is epilepsy. In another highly preferred embodiment, treatment with the compound or agent enhances cognitive function.

In a preferred embodiment, the agent or compound is LEV or an analog or derivative thereof. In another preferred embodiment, the compound or agent binds to the levetiracetam binding site of an SV2 protein. In still another preferred embodiment, the compound or agent competes with levetiracetam or an analog or derivative thereof for binding to the levetiracetam binding site. In yet another preferred embodiment, agents of the invention for the treatment of neurological disorders include N-alkylated 2-oxo-pyrrolidine derivatives, N-

alkylated 2-oxo-piperidiny] derivatives, and N-alkylated 2-oxo-azepany] derivatives as described above.

In a preferred embodiment, the compound or agent is an anti-SV2 antibody or fragment thereof, including those that bind to the levetiracetam binding site of SV2 protein and may be a polyclonal antibody or a monoclonal antibody. In related preferred
5 embodiments, the antibody fragment is an Fab fragment, Fab' fragment, F(ab')₂ fragment or an scFv fragment, whereas the monoclonal antibody is a chimeric antibody, humanized antibody, or a human antibody.

As used herein, a subject can be any mammal, so long as the mammal is in need of
10 modulation of function or activity of a SV2 protein mediated by a compound or agent identifiable by a method of the invention. The term mammal is defined as an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects.

As used herein, an "effective amount" is an amount of a substance, compound or
15 agent which is effective to inhibit, reduce, ameliorate, modulate or control at least one symptom or effect of a disease, condition or another administered substance, compound or agent either *in vivo*, *ex vivo*, or *in vitro*. Further as used herein, an "effective amount" is an amount of a substance, compound or agent which is effective to enhance at least one cognitive function *in vivo*.

As used herein, an agent is said to modulate a neurological disorder when the agent
20 reduces the degree or severity of at least one symptom the neurological disorder. For instance, seizures in epilepsy may be prevented; the amplitude, magnitude or severity of seizures may be reduced, or the frequency of the occurrence of seizures may be reduced by the administration of compounds or agents which up- or down-regulate or modulate in some
25 way the expression or at least one activity of a SV2 protein of the invention.

The compounds or agents identified by the methods of the present invention can be provided alone, or in combination with other compounds or agents that modulate a particular pathological process. For example, a compound or agent of the present invention can be administered in combination with other known drugs. As used herein, two agents are said to
30 be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time. In a

particular embodiment of the invention, the compounds or agents identified by the methods of the present invention can be provided in combination with compounds or agents that modulate GABAergic pathways in the brain. In another embodiment, the compounds of the invention are administered together with amantadine for combined treatment of L-DOPA
5 and tardive dyskinesia.

The compounds or agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, epidural, transdermal, topical, or mucosal routes, or combinations thereof. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of
10 treatment, and the nature of the effect desired. Drug solubility and the site of absorption are factors which should be considered when choosing the route of administration of a therapeutic agent.

The present invention further includes compositions containing one or more compounds or agents which modulate expression or at least one activity of a SV2 protein of
15 the invention. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise about 0.1 to about 100 mg/kg body weight. The preferred dosages comprise about 5 to about 80 mg/kg body weight. More preferred dosages comprise about 10 to about 60 mg/kg body weight. The most preferred dosages comprise about 20 to about 40 mg/kg body weight.

20 In addition to the pharmacologically active agent, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action.

The compounds or agents may be formulated for parenteral administration by
25 injection, *e.g.*, by bolus injection or continuous infusion. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example,
30 ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl

cellulose, sorbitol, and/or dextran. Liposomes can also be used to encapsulate the agent for delivery into the cell. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

Mucosal routes of administration include, but are not limited to, oral, rectal and nasal administration. Preparations for mucosal administrations are suitable in various formulations. If the compound or agent is water-soluble, then it may be formulated in an appropriate buffer, for example, phosphate buffered saline or other physiologically compatible solutions, preferably sterile. Alternatively, if the resulting complex has poor solubility in aqueous solvents, then it may be formulated with a non-ionic surfactant such as Tween, or polyethylene glycol. Thus, the compounds and their physiologically acceptable solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral, or rectal administration or, in the case of tumors, directly injected into a solid tumor. For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

For oral administration, the pharmaceutical preparation may be in liquid form, for example, solutions, syrups or suspensions, or may be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-

hydroxybenzoates or sorbic acid). The pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well-known in the art. Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

The compounds or agents may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds or agents may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophilic drugs.

In practicing the methods of this invention, the compounds or agents of this invention may be used alone or in combination, or in combination with other therapeutic or diagnostic agents. In certain preferred embodiments, the compounds of this invention may be co-administered along with other compounds or agents typically prescribed for these conditions according to generally accepted medical practice such as anticonvulsives. The compounds of this invention can be utilized *in vivo*, ordinarily in mammals, such as humans, sheep, horses, cattle, pigs, dogs, cats, rats and mice, or *in vitro*.

IX. Gene Therapy

SV2 proteins used in treatment can be generated endogenously in the subject, in treatment modalities often referred to as "gene therapy." In a specific embodiment, nucleic acids comprising sequences encoding SV2 proteins or functional derivatives thereof, are administered to treat, inhibit or prevent a neurological disease or disorder associated with aberrant synaptic function, by way of gene therapy. Gene therapy refers to therapy performed by the administration to a subject of an expressed or expressible nucleic acid. In this embodiment of the invention, the nucleic acids produce their encoded SV2 protein that mediates a therapeutic effect.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel *et al.*, Clinical Pharmacy 12:488-505. (1993); Wu and Wu, Biotherapy 3:87-95. (1991); Tolstoshev, Ann. Rev. Pharmacol. Toxicol. 32:573-596. (1993); Mulligan, Science 260:926-932. (1993); and Morgan and Anderson, Ann. Rev. Biochem. 62:191-217. (1993); TIBTECH11(5):155-215. (1993)). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel *et al.*, eds., Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); and Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990).

In a preferred aspect, the compound comprises nucleic acid sequences encoding an SV2 protein, said nucleic acid sequences being part of expression vectors that express the SV2 protein or fragments or chimeric proteins thereof in a suitable host. In particular, such nucleic acid sequences have promoters operably linked to the SV2 protein coding region, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another particular embodiment, nucleic acid molecules are used in which the SV2 protein coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the antibody nucleic acids (Koller *et al.*, Proc. Natl. Acad. Sci. USA 86:8932-8935. (1989); Zijlstra *et al.*, Nature 342:435-438. (1989)).

Delivery of the nucleic acids into a patient may be either direct, in which case the

patient is directly exposed to the nucleic acid or nucleic acid-carrying vectors, or indirect, in which case, cells are first transformed with the nucleic acids *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

5 In a specific embodiment, gene therapy vectors can be delivered *in vivo* to a subject by, for example, intravenous injection, local administration (see U.S. Pat. No. 5,328,470) or by stereotactic injection (see *e.g.*, Chen *et al.* Proc. Natl. Acad. Sci. USA 91:3054-3057. (1994)). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the
10 gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

Direct administration of the nucleic acid sequences for expression of encoded SV2 protein *in vivo* can be accomplished by any of numerous methods known in the art, *e.g.*, by
15 constructing them as part of an appropriate nucleic acid expression vector and administering them so that they become intracellular, *e.g.*, by infection using defective or attenuated retroviral vectors or other viral vectors (see U.S. Pat. No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in
20 liposomes, microparticles, or microcapsules, or by administering them in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see, *e.g.*, Wu *et al.*, J. Biol. Chem. 262:4429-4432. (1987)) (which can be used to target cell types specifically expressing the receptors), etc. In another embodiment, nucleic acid-ligand complexes can be formed in which the ligand comprises a
25 fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, *e.g.*, PCT Publications WO 92/06180 dated Apr. 16, 1992 (Wu *et al.*); WO 92/22635 dated Dec. 23, 1992 (Wilson *et al.*); WO 92/20316 dated Nov. 26, 1992 (Findeis *et al.*); WO 93/14188 dated Jul. 22, 1993 (Clarke *et al.*); and WO 93/20221 dated Oct. 14, 1993 (Young)). Alternatively, the nucleic
30 acid can be introduced intracellularly and incorporated within host cell DNA for expression,

by homologous recombination (Koller *et al.*, Proc. Natl. Acad. Sci. USA 86:8932-8935. (1989); Zijlstra *et al.*, Nature 342:435-438. (1989)).

In a specific embodiment, viral vectors that contain nucleic acid sequences encoding an SV2 protein of the invention are used. For example, a retroviral vector can be used (see
5 Miller *et al.*, Meth. Enzymol. 217:581-599. (1993)). These retroviral vectors have been to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. The nucleic acid sequences encoding the antibody to be used in gene therapy are cloned into one or more vectors, which facilitates delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen *et al.* (Biotherapy
10 6:291-302. (1994)), which describes the use of a retroviral vector to deliver the *mdr1* gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes *et al.*, J. Clin. Invest. 93:644-651. (1994); Kiem *et al.*, Blood 83:1467-1473. (1994); Salmons *et al.*, Human Gene Therapy 4:129-141. (1993); and Grossman *et al.*, Curr. Opin. in Genetics and
15 Devel. 3:110-114. (1993).

Adenoviruses are other viral vectors that can be used in gene therapy. In a preferred embodiment, adenovirus vectors are used. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky *et al.*, Current Opinion in Genetics and Development 3:499-503. (1993) present a review of adenovirus-based gene therapy. Bout *et al.*,
20 Human Gene Therapy 5:3-10. (1994) demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld *et al.*, Science 252:431-434. (1991); Rosenfeld *et al.*, Cell 68:143-155. (1992); Mastrangeli *et al.*, J. Clin. Invest. 91:225-234. (1993); PCT Publication WO 94/12649; and Wang, *et al.*, Gene Therapy 2:775-783. (1995).

25 Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh *et al.*, Proc. Soc. Exp. Biol. Med. 204:289-300. (1993); U.S. Pat. No. 5,436,146).

Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a
30 selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a

patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including, but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see, *e.g.*, Loeffler and Behr, *Meth. Enzymol.* 217:599-618. (1993); Cohen *et al.*, *Meth. Enzymol.* 217:618-644. (1993); Cline, *Pharmac. Ther.* 29:69-92. (1985)) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, endocrine cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, *etc.* In a preferred embodiment, the cells used for gene therapy is autologous to the patient.

In an embodiment in which recombinant cells are used in gene therapy, nucleic acid sequences encoding an antibody are introduced into the cells such that they are expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained *in vitro* can potentially be used in accordance with this embodiment of the present invention (see *e.g.* PCT Publication WO

94/08598, dated Apr. 28, 1994; Stemple and Anderson, Cell 71:973-985. (1992); Rheinwald, Meth. Cell Biol. 21A:229. (1980); and Pittelkow and Scott, Mayo Clinic Proc. 61:771. (1986)).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

X. Uses for Biotinylated Ligands

The present invention provides nonradioactive labelled SV2A/LBS ligands containing a biotin tag. Such biotinylated ligands are useful in screening assays with no radioactive waste and higher throughput.

As an example, biotinylated derivatives of SV2A/LBS ligands can be used in screening assays (*e.g.* binding) with native brain membranes or SV2 expressed in cell lines for the discovery of more potent structures. The amount of biotin tag bound to SV2A can be quantified using streptavidin-fluorescein or avidin derivatives.

Biotinylated ligands are also useful for assessing the conformational state of SV2 after solubilization, immunoaffinity purification, and chromatography.

Moreover, the present invention provides photoactivable versions of the ligands for labeling and detection in biological samples. The photoactivable biotinylated ligands may also be used to localize and purify SV2 from tissues, isolated cells, subcellular fractions and membranes. The photoactivable biotinylated ligands could also be used for SV2 cross-linking and identification of binding domains of LBS ligands.

XII. Solubilizing SV2 and Affinity Purification

The present invention provides a method for solubilizing SV2/LBS proteins comprising treating membranes with a detergent. The membrane proteins solubilized by the present method remain active as evaluated by binding assays and protein-protein interaction studies.

Briefly, the method comprised incubating membranes, as an example rat brain membranes, in solubilization buffer containing the detergent n-dodecyl- β -D-maltoside for

about two hours at about 4°C. The incubated solution was subsequently centrifuged to collect the soluble SV2 protein, specifically the SV2A protein, from the supernatant. Presence of the soluble SV2A protein in the supernatant was confirmed by western blot analysis using anti-SV2A antibodies. The binding activity of the soluble SV2A protein in the supernatant was determined through binding experiments with ligands known to bind SV2A, such as levetiracetam and ucb 30889.

Other detergents such as the analogs of n-dodecyl-β-D-maltoside, for example, n-octyl, n-nonyl, n-decyl, n-undecyl-β-D-maltoside could also be used. In fact, preliminary data confirmed that the soluble protein obtained from solubilizing membranes with these detergents retains its binding activity.

The present invention also provides a method of affinity purification of the soluble SV2 protein and identification of putative SV2A partners. Briefly, affinity purification comprised incubating the supernatants from the solubilized membranes with anti-SV2A antibodies overnight at about 4°C. The mixture was then incubated by rotation with protein A-Sepharose beads in buffer for about an hour at about 4°C. The resin was washed several times with an appropriate buffer and the fractions containing the immunopurified SV2A protein were collected.

To detect the presence of binding partners of SV2A after affinity purification, a western blot analysis of the immunopurified fractions was performed to detect the presence of synaptotagmin (Figure 22)

The present invention provides a method to purify a membrane-associated protein comprising solubilizing a membrane sample containing the protein with a detergent to form a solubilized complex and isolating the solubilized complex in a functional form. The detergent could be n-dodecyl-β-D-maltoside or derivatives thereof. The protein can then be isolated using an immunoaffinity technique.

The protein purified by the present method can be used to perform structural studies on the protein such as NMR, X-ray crystallography, Infrared spectroscopy, Circular dichroism and other methods well known in the art. The present invention also provides a method of performing SV2 protein interaction studies and for detecting peptides, molecules, and compounds that inhibit or promote the interactions between SV2 and a putative partner. The present invention can be used to identify SV2 binding partners.

The present invention could be used to solubilize SV2A, SV2B and SV2C membrane associated proteins and to affinity purify them for structural studies and for identifying binding partners.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

Example 1. Development of a Levetiracetam Analog for Binding Studies

LEV has been shown to bind to a specific binding site located preferentially in the brain (levetiracetam binding site or LBS : Noyer *et al.*, Euro. J. Pharmacol. 286:137-146.

(1995); Gillard *et al.* 2003)). However, [³H]LEV displayed only micromolar affinity for this site, making it unsuitable for in depth characterization. This example describes the binding properties of [³H]ucb 30889, (2S)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide, an analogue of levetiracetam. Binding experiments were conducted on crude rat brain membranes at 4°C as described in Noyer *et al.* (Euro. J. Pharmacol. 286:137-146 (1995)).

Incubation time for equilibrium studies was 120 min. For kinetic and competition studies, [³H]ucb 30889 (30 Ci / mmol) was used at a concentration of 1.3 nM in 0.5 ml of a Tris-HCl (pH 7.4) buffer containing 2 mM Mg²⁺. Localization of the LBS in brain substructures was assessed by autoradiography on 25 µm thick slices incubated under similar conditions. Slides were then washed twice for 10 min at 4°C in 50 mM Tris-HCl (pH 7.4) containing 0.5% BSA, dried and exposed for 3 weeks to [³H]Hyperfilm at -20°C. Non-specific binding (NSB) was determined by the inclusion of 1 mM LEV during the incubation period.

Figure 1 shows that [³H]ucb 30889 binds reversibly to LBS in rat brain cortex. Binding kinetics were biphasic: half-times for association and dissociation were respectively, 3 ± 2 min and 4 ± 1 min for the fast component (25 to 50 % of the sites), and 47 ± 13 min and 61 ± 15 min for the slow component. At 25°C, kinetics increased dramatically and only one component remained.

Figure 2 shows that the saturation binding curves of [3 H]ucb 30889 were compatible with the labeling of a homogeneous population of binding sites. K_D and B_{max} were respectively 42 ± 10 nM and 5054 ± 704 fmol / mg protein. The B_{max} being similar to the value estimated using [3 H]levetiracetam as radioligand in similar membrane preparations
5 (4718 \pm 413 fmol / mg protein).

Specific binding could not be detected in the peripheral tissues examined (Figure 3). The limit of detection under the experimental conditions (150 μ g of protein / assay and 1.3 nM of radioligand) was a B_{max} of 200 fmol / mg protein. This suggests that there are at least 25 times more binding sites in the cerebral cortex compared to the periphery.

10 Competition binding curves showed that ucb 30889 binds to LBS with about 10 fold higher affinity than LEV (Figure 4). The pK_i of ucb 30889 (7.1 ± 0.2) agrees well with the K_D of [3 H]ucb 30889 as determined by the saturation binding curve (Figure 2). pIC_{50} values for a variety of levetiracetam analogues and other compounds known to interact with the LBS, such as pentylenetetrazol or bemegride (Noyer *et al.*, 1995), were identical whether
15 obtained with [3 H]ucb 30889 or [3 H]levetiracetam (Figure 5).

Rat brain sections incubated with [3 H]ucb 30889 (Figure 6) show that LBS labeled by [3 H]ucb 30889 are diffusely localized throughout the brain and that this binding can be inhibited by levetiracetam at concentrations equivalent to those observed in *in vitro* binding (Figure 4).

20 This example demonstrates through competition binding studies and tissue distribution that ucb 30889 and LEV are both labeling the same sites, namely the LBS which is localized throughout the central nervous system. Compared to LEV, ucb 30889 binds to the LBS with 10 fold higher affinity and with a very low non specific binding. These criteria along with suitable binding kinetics at 4°C made it possible to use this radioligand to perform
25 autoradiography binding studies on brain slices (Figure 6) and to show the anatomical distribution of LBS in rat brain.

Example 2. Cellular and Subcellular Distribution of the LBS

To identify and characterize the LBS *in situ*, [3 H]ucb 30889 was used to map the
30 LBS within the brain and to study both its cellular and subcellular distribution. For rat brain autoradiography, 25 μ m slices were incubated with 1.3 nM [3 H]ucb 30889 for 120 min at 4

°C in 50 mM Tris-HCl buffer (pH 7.4). Binding assays with rat brain membranes and various neuronal cell lines were performed under similar conditions. Non-specific binding was determined by the inclusion of 1 mM levetiracetam in the assay. For photolabeling, membranes were incubated with 40 nM [³H]ucb 30889 for 120 min at 4°C in the same buffer, followed by irradiation with UV-light for 30 min (Fuks *et al.*, Eur. J. Pharmacol. 478:11-19 (2003)).

For rat brain autoradiography, 25 µm slices were incubated with 1.3 nM [³H]ucb 30889 for 120 min at 4 °C in 50 mM Tris-HCl buffer (pH 7.4). Figure 7 shows that ucb 30889 binding sites are heterogeneously distributed in the rat brain. While there is no apparent binding in the white matter there is a high level of binding in the dentate gyrus, the superior colliculus, several thalamic nuclei and in the molecular layer of the cerebellum. Binding is less pronounced in the cerebral cortex, the hypothalamus and the striatum. Abbreviations: cc, corpus callosum; Aca, anteria commissure; ic, internal capsule; Mtg, mamillotegmental tractus; Mt, mammillothalamic tractus; ML, molecular layer; Hi, hippocampus; DG, dentate gyrus; sc, superior colliculus; CG, central grey; Pu, caudate putamen; Pv, paraventricular nucleus; MG, geniculate nuclei; Po hy, posterior hypothalamic areas; Hb, habenula; Pi, piriform cortex.

[³H]ucb 30889 binding in cerebellar granule neurons and PC12 cells showed high levels of specific binding (Table 1). The K_d being similar to the value measured in rat cerebral cortex (42 nM; see Example 1). The same specific binding site could not be detected in primary astrocytes and in a range of CNS-related cell lines and non neuronal cell lines. Abbreviation: nd, not detected.

TABLE 1

Density and affinity of [3 H]ucb 30889
binding in various cell types

5	Cell type	B _{max}	K _d
	Rat cerebellar granule neurons	0.7 pmol/mg protein	59 nM
	Mouse cortical neurons	1.4 pmol/mg protein	34 nM
	Mouse cortical astrocytes nd	nd	
10	PC12	1.4 pmol/mg	40nM
	SK-N-SH	nd	nd
	NG108-15	nd	nd
	N1E-115	nd	nd
	HCN-1a	nd	nd
15	CHO-K1	nd	nd
	COS-7	nd	nd

Rat brain membranes were separated by differential centrifugation (Figure 8).

20 Binding to LBS (8A), muscarinic (8B), NMDA (8C) and peripheral benzodiazepine (8D) receptors was determined using [3 H]ucb 30889, [3 H]NMS, [3 H]MK801 or [3 H]PK11195, respectively. This study shows that the levetiracetam binding site is present in crude synaptosomes (P2), microsomal membranes (P3) and is enriched in synaptic vesicles (LP2). In contrast, the other studied receptors are not more abundant in LP2 compared to P2 or P3. P1 is a low speed pellet containing nuclei and large debris.

25 A fractionation onto a sucrose gradient was used to isolate the subcellular compartments from crude synaptosomes. The LBS was found in purified synaptic membranes but was not present in the 1.2 M sucrose pellet containing the purified mitochondrial fraction (Figure 9). As a control for the purity of the subcellular fractions, the distribution of the muscarinic and the peripheral benzodiazepine receptors was also analyzed.
30 Data are expressed as percentage of the total specific binding.

Crude synaptosomes (P2 fraction) were preincubated with 40 nM [3 H]ucb 30889, then

irradiated with UV light and washed. At 0 min 1 mM levetiracetam was added and aliquots were counted at the indicated times (Figure 10A). Nonspecific binding (open symbol) was determined using 1 mM levetiracetam. Figure 10B shows the same experiment, but performed in the absence of UV light irradiation. These results indicate that during UV irradiation the radioligand inserts covalently in the binding domain of the LBS.

Photoaffinity labeling was performed in the absence or in the presence of 1 mM levetiracetam. The proteins were resolved by SDS-PAGE using an acrylamide concentration of 7.5 % (w/w) and the radioactivity was assessed in each slice of the gel. The major site of incorporation occurs at a molecular weight of 93,000 (Figure 11) (Fuks *et al.*, 2003)

In this example it is shown that the [³H]ucb 30889 binding site in rat brain has a unique profile of distribution and does not appear to correlate with any specific neurotransmitter system that is typically associated with epilepsy. This novel binding site is restricted to neuronal cell types and several brain areas. This novel radioligand can be used as a photoaffinity label and binds covalently to a membrane protein of high molecular weight which is mainly located in synaptic vesicles.

Example 3. The LBS is on SV2A

In this example, the biochemical characterization of LBS in rat brain led to studies to identify potential candidate LBS proteins for cloning and binding characterization. Based on the integral membrane nature of the protein, brain specific expression, apparent size, and synaptic vesicle localization, the SV2 protein family was analyzed as a candidate for localization of the LBS. Accordingly, SV2 proteins were cloned and assayed for binding of LBS ligands.

Materials: Levetiracetam and derivatives were synthesized at UCB Pharma (Braine-l'Alleud, Belgium). [³H]ucb 30889, (2S)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide (32 Ci/mmol), was custom labelled by Amersham Biosciences (Roosendaal, The Netherlands). The monoclonal antibody against SV2 proteins developed by Buckley and Kelly (Buckley *et al.*, J. Cell. Biol., 100, 1284-94 (1985)) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA

52242. This antibody is cross-reactive against all three SV2 isoforms, SV2A, SV2B, and SV2C.

Wild-type and Knockout Mouse Binding Experiments

SV2A knockout mice have been previously reported (Crowder *et al.* Proc. Natl. Acad. Sci. U.S.A. 96, 15268-73 (1999)). The generation of SV2B knockouts will be reported elsewhere. SV2B knockouts were bred with animals heterozygous for the SV2A gene disruption to produce SV2A^{+/}-SV2B^{-/-} breeders which were used to generate SV2A/B knockouts. Wild type C57-B16 and SV2 KO mouse brain membranes were prepared for binding assays and the binding reaction was performed as described previously with slight modifications (Gillard *et al.*, Eur. J. Pharmacol. In Press (2003)). Frozen whole brains were homogenized (10 % w/v) in 20 mM Tris-HCl buffer (pH 7.4) containing 250 mM of sucrose (buffer A). The homogenates were spun at 30,000 x g at 4°C for 15 min and the pellets resuspended in the same buffer. After incubation at 37°C for 15 min, the membranes were washed 2 times using the same centrifugation protocol. The final pellets were resuspended in buffer A and stored in liquid nitrogen. Thawed brain membrane proteins (0.1 mg/assay) were incubated 120 min at 4°C in 0.5 ml of a 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM MgCl₂, and [³H]ucb 30889 (1.8 nM). At the end of the incubation period, the membrane-bound radioligand was recovered by rapid filtration through GF/C glass fiber filters pre-soaked in 0.1% polyethyleneimine. The membranes were washed with 8 ml of ice-cold Tris buffer (pH 7.4). The total filtration procedure did not exceed 10 s per sample. The filters were dried and the radioactivity determined by liquid scintillation. pIC₅₀s determination was performed by computerized non-linear curve fitting methods (Graphpad Prism[®] software, San Diego, CA).

For Western blot experiments, aliquots of brain homogenates from the wildtype and knockout animals were extracted at room temperature with SDS-PAGE sample buffer containing BME. Equivalent amounts of each sample (approx. 10µg total protein) were loaded on a 4-12% Tris-Glycine NOVEX gradient gel (Invitrogen Life Sciences) and separated. After transfer to a nitrocellulose membrane and blocking, the blot was probed with a monoclonal cross-reactive to all SV2 proteins (Buckley *et al.*, J. Cell. Biol., 100, 1284-94 (1985)), and an HRP-anti-mouse secondary antibody was used to label the primary. The

blot was developed with luminescent horseradish peroxidase reagents and photographed.

Binding Experiments with [³H]ucb 30889 against heterologously expressed hSV2A

For binding experiments on confluent, transfected cells (Figure 17), cells in 24 well
5 plates were slowly cooled to 4°C and rinsed once with cold phosphate-buffered saline (PBS).
PBS was aspirated and binding reagents were added in PBS. In binding experiments, [³H]ucb
30889 was added to all wells at 1.8 nM, in the presence or absence of differing amounts of
unlabelled inhibitors. The cells were incubated at 4°C for 2h and the assay was terminated by
rinsing the cells 3x rapidly with ice-cold PBS. After a final aspiration, 200µl of 0.1N NaOH
10 was added to lyse the cells, and the samples were counted in scintillation fluid on a beta
counter.

For binding experiments on previously frozen transfected COS-7 cells (Figure 18), 2
to 3x10⁴ cells were incubated 120 min at 4°C in 0.2 ml of a RPMI-HEPES 25 mM solution
containing [³H]ucb 30889 (1.8 nM) and increasing concentrations of unlabelled competing
15 drugs. The termination of the binding reaction by filtration and radioactivity counting was
performed as described above.

Audiogenic Seizure Mouse Model

Anti-seizure activity of LEV and analogues were assessed in sound-susceptible mice
20 by exposing the mice to acoustic stimuli of 90-db, 10 to 20-kHz for 30sec, 60 min following
intraperitoneal pretreatment. The reported ED₅₀ values were obtained from testing of 4 to 8
groups (n=10) administered different doses and reflect the potency of the compounds for
inhibiting clonic convulsions.

Human SV2A was cloned from a human fetal brain cDNA library as a 3609 bp PCR
25 product comprising the coding region and significant flanking regions from the transcript.
Using a vector containing the SV2A coding region plus significant flanking DNA as a source,
the coding region was PCR amplified without the flanking regions. This product was cloned
into a GATEWAY (Invitrogen) donor vector for ease of subcloning. Only the use of a
cloning vector with strong transcription stop sites directly upstream of the cloning site
30 resulted in successful cloning of coding-region only SV2A cDNA. This suggests that this
product may be toxic to *E coli*, even in small amounts. Sequencing of the final pDONR
GATEWAY SV2A clone showed that it had 2 mutations: one silent, and one a Leu-to-Pro

mutation. The non-silent mutation was corrected and sequencing confirmed that the correct, full length human SV2A coding sequence was cloned.

The human SV2A coding region was transferred from the pDONR GATEWAY cloning vector to a pDEST 12.2 Gateway expression vector. This vector has a CMV promoter driving the introduced gene, and an SV40 ori, which allows very high levels of replication in the COS-7 cell line, which contains the large T antigen. In addition, the human SV2A coding region was transferred into a pDEST 40 Gateway expression vector. This vector is very similar to the 12.2 vector above, with a CMV promoter driving expression of hSV2A, and an SV40 ori, and a Neomycin resistance gene.

Initial tests of SV2A expression using the pDEST 12.2 vector was performed in the COS-7 cell line, which had previously been demonstrated successful expression of SV2 proteins. The COS-7 cell line was tested for ^3H -30889 binding, with no binding above background observed, and thus no significant, measurable presence of the Levetiracetam binding site (LBS). In addition, a PC12 cell line subclone, PC12a, which is low in LBS, was used to establish a pool of PC12 cells expressing hSV2A under stable antibiotic selection.

Lipofectamine 2000 (Invitrogen) transfection reagent was used to transfect DNA into 90% confluent COS-7 cells. Also, the same reagent was used to transfect the hSV2A containing vector into the PC12a cell line, and selecting for antibiotic resistance. Anti-SV2A polyclonal antibody (CalBiochem) was used to test for expression in either transfected COS-7 cells, or transfected PC12a cells, of the SV2A product. Lysates of the COS-7 cells were collected at 18 hrs after transfection on an SDS-PAGE gel, transferred to a membrane, and probed with a polyclonal antibody against SV2A, in comparison to crude rat brain membranes (Figure 12A). Also shown are lysates from a non-transfected COS-7 cells, non-transfected PC12a cells (low in LBS), PC12bs cells (high in LBS), or PC12a cells transfected with hSV2A (Figure 12B). No labeling of protein bands is observed in the untransfected COS-7 control, while the transfected COS-7 cells show multiple bands, with most density in the range of 80-120 kD, perhaps due to multiple glycosylation states of the expressed protein. In addition, SV2A immunoreactivity is present in the PC12bs and PC12a/hSV2A samples, but largely absent in the low LBS PC12a cells (Figure 12B).

In a binding experiment, specific binding was measured of [^3H]ucb 30889 to COS-7 cells that have either been transfected with SV2A-12.2, or as controls, a β -gal expressing

vector, or cells that have not been transfected (Figure 13). Triplicate wells of a 24-well plate were incubated with either 1 nM [^3H]ucb 30889 (labeled "Hot"), or [^3H]ucb 30889 plus an excess of cold Levetiracetam (50 μM) (labeled "Hot + Cold"). The cells were incubated at 4 $^{\circ}\text{C}$ for 2 hours, and then washed rapidly with ice-cold PBS. The cells were lysed on the plate, transferred to scintillation vials with scintillation fluid and counted for ^3H decay emission. These results show that COS-7 cells transfected with SV2A have acquired the capability to specifically bind [^3H]ucb 30889. In identical intact cell binding experiments using PC12bs cells, known to express the LBS, a 1.5 to 2-fold difference in CPM between the 'hot' and 'hot+cold' samples is typically seen, as compared to the 5-fold difference seen here.

Further studies characterized the binding of [^3H]ucb 30889 to SV2A expressed in COS-7 cells in more detail. COS-7 cells were transfected in a 24-well plate and assayed for binding as above. A series of concentrations of either Levetiracetam or cold 30889 were added in order to generate IC_{50}s for these compounds against SV2A expressed in COS-7 cells (Figure 14). These results indicate that SV2A is functionally equivalent with the binding site for Levetiracetam that has been observed in rat brain and PC12 subclones. The correlation between LBS binding affinity and the anti-seizure properties of Levetiracetam and its analogues, taken together with the preceding observation, provide support that the synaptic vesicle protein SV2A is not only the native binding site for the anti-epileptic compound Levetiracetam, but suggests a link between the function and modulation of the synaptic vesicles by Levetiracetam and its anticonvulsant properties.

In a separate experiment, heterologous expression experiments were performed to confirm that SV2A alone is solely responsible for the brain binding of LEV. Human SV2A was transiently expressed in the COS-7 cell line, as verified by Western analysis (data not shown), and observed binding to [^3H]ucb 30889 that is displaced by excess LEV (Figure 17A). No binding under identical conditions to either untransfected COS-7 cells, or COS-7 cells transfected with a vector encoding β -galactosidase. In experiments testing the ability of unlabeled compounds to displace [^3H]ucb 30889 from hSV2A expressed in COS-7 cells, the affinities of ucb 30889, LEV, and LEV's enantiomer, ucb L060, show the same rank order, and similar values (Figure 17B), to those previously reported in studies with rat brain ((Noyer *et al.*, Eur. J. Pharmacol. 286, 137-146 (1995); Gillard *et al.*, 2003). Critically, ucb L060 binds with significantly less affinity to hSV2A than does LEV, which is a key characteristic

of the binding site in brain (Noyer *et al.*, Eur. J. Pharmacol. 286, 137-146 (1995); Gillard *et al.*, 2003). In addition, the binding of [³H]ucb 30889 against both hSV2B and hSV2C expressed in the transient COS-7 system were tested. The results indicate no binding above background (data not shown), consistent with the results from knockout mouse binding studies.

5 Testing the binding of LEV and several analogs to hSV2A expressed in COS-7, revealed that pIC₅₀s are highly correlated ($r^2=0.98$) with the values obtained in mouse brain (Figure 18A) and rat brain extracts (data not shown). There was also a clear correlation between the affinities of these compounds for hSV2A in COS-7 and the potency of their antiseizure protection in the mouse audiogenic model of epilepsy ($r^2=0.84$) (Figure 18B).

10 This data is consistent with a previous report of a correlation between binding of LEV analogs in rat brain and potency in the same model (Noyer *et al.*, Eur. J. Pharmacol. 286, 137-146 (1995)). The binding of other AEDs, including valproate, carbamazepine, phenytoin, ethosuximide, felbamate, gabapentin, tiagabine, vigabatrin and zonisamide was also investigated. None of the AEDs at concentrations up to 100 μ M, competed with [³H]ucb
15 30889 for binding to SV2A (data not shown). This confirms previous binding studies of AEDs against the LEV binding site in rat brain (Noyer *et al.*, Eur. J. Pharmacol. 286, 137-146 (1995); Gillard *et al.*, Eur. J. Pharmacol. 2003)).

20 **Example 4. Assays for Compounds Which Modulate Neurological Disorders, Endocrinopathy and Hormonal Diseases**

In order to identify compounds or agents which modulate neurological disorders associated with synaptic function and endocrinological disorders, studies were undertaken to identify additional compounds which compete with LEV and ucb 30889 for binding to the LBS of a SV2 protein.

25 SV2A transfected COS-7 cells as disclosed in Example 3 are exposed to a potential binding partner or agent. Control cells are exposed to vehicle only, or are exposed to unlabeled ucb 30889 or LEV. Following this exposure, the cells are then incubated with [³H]ucb 30889, as in Example 3, cells are incubated at 4 °C for 2 hours, and then washed rapidly with ice-cold PBS. The cells are lysed, transferred to scintillation vials with
30 scintillation fluid and counted for ³H decay emission.

Compounds which are found to compete with ucb 30889 for binding to the LBS are

subject to further analysis for the ability to modulate seizures in audiogenic-susceptible mice. Audiogenic-susceptible mice are administered an amount of the compound which is comparable to an effective amount of LEV. As a control, identical audiogenic-susceptible mice are administered an effective amount of LEV, or a compound which does not modulate seizures, such as piracetam.

Example 5. Biotinylated Ligands as Tools to Screen Chemical Libraries and Characterize the SV2 Proteins

The present invention discloses a method of using novel biotinylated ligands as tools to screen chemical libraries and characterize SV2 proteins. The present invention provides nonradioactive-labelled SV2A/LBS ligands containing a biotin tag for screening purposes with no radioactive waste and higher throughput. The present invention also provides a photoactivable version for labelling and SV2A/LBS detection in biological samples.

In this example, the binding of ucb-101282-1 to SV2A/LBS was characterized in rat brain membranes. This molecule is a biotinylated derivative of ucb 30889 (Figure 19). This ligand had a pK_i of 6.3 (n=2) in rat brain membranes which was equivalent to the affinity reported for L059 (Figure 20). This ligand was also designed to cross-link the biotin tag to the LBS/SV2A with an azidophenyl motif capable of forming a covalent complex with the protein upon UV light irradiation.

Example 6. Method for Solubilizing SV2A and Affinity Purification

The present invention discloses a method of solubilizing SV2A and affinity purification. The method comprises solubilizing SV2A/LBS proteins which includes treating the membrane with a detergent. The method maintains the activity of the membrane proteins after solubilization as evaluated in binding assays and protein-protein interaction studies.

Preparation of Soluble SV2A and Quantitation by Binding Assay

The rat brain membranes were diluted in a solubilization buffer (Tris-HCl 20 mM pH 7.4, 0.25 M sucrose, protease inhibitors Complete Roche) containing n-dodecyl-β-D-maltoside 15 mM and incubated for two hours at 4°C. Subsequently, the solution was centrifuged at 4°C for one hour at 100,000 g. The soluble SV2A was found in the

supernatant as detected by western blot using anti-SV2A antibodies (Figure 21A). The supernatants were incubated with [³H]ucb 30889 as described. Binding experiments indicated that the specific binding is due to a soluble form of SV2A. In order to detect the specificity of the SV2A binding, the ability of levetiracetam and ucb 30889 to specifically bind to the soluble SV2A was examined. The affinities of the molecules were equivalent to that exhibited by ligands to the native membrane (Figure 21B). Scatchard analysis indicates that the K_D for [³H]ucb 30889 for to the SV2A in native membrane is 30nM, while that for the soluble protein is 82 nM (Figure 21C). Thus, the binding properties of the soluble SV2A are similar to the membrane-bound native form indicative that the soluble protein maintains its native structural conformation in n-dodecyl-β-D-maltoside.

Affinity Purification of the Soluble SV2A and Identification of Putative SV2A Partners

Supernatants from solubilized membranes were incubated with anti-SV2A antibodies overnight at 4°C. The mixture was rotated with protein A-Sepharose beads for 1 hour at 4°C in Tris-HCl 20 mM pH 7.4, 0.25 M sucrose, protease inhibitors Complete (Roche). The resin was washed several times and the collected fractions contained immunopurified SV2A (Figure 22). As explained above, SV2A is maintained in its native conformation after solubilization in n-dodecyl-β-D-maltoside. Therefore, since synaptotagmin is a well known partner of SV2A, the immunopurified fractions were tested to determine whether synaptotagmin was still associated to SV2A after the purification procedure. Western analysis of the immunopurified fractions confirmed the presence of synaptotagmin associated to soluble SV2A, while the isoform SV2B was not detected. Thus, the solubilization and purification procedure can be used to perform SV2A-protein interactions studies.

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents, patent applications and publications referred to in this application are herein incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. A method of treating a neurological disorder associated with synaptic vesicle function, endocrinopathy or hormonal diseases, comprising administering a compound or agent that modulates a function or activity of an SV2 protein.
5
2. A method of claim 1, wherein the neurological disorder is selected from the group consisting of seizure, epilepsy, Parkinson's disease, Parkinson's dyskinesias, migraine, Alzheimer's disease, neuropathic pain, essential tremor, cognitive disorders, and movement disorders.
10
3. A method of claim 1, wherein the compound or agent binds to the levetiracetam binding site of an SV2 protein.
- 15 4. A method of modulating at least one function or activity of a SV2 protein in a cell, comprising exposing the cell to a compound or agent that binds to the levetiracetam binding site of the SV2 protein.
5. A method of claim 4, wherein the compound or agent modulates the binding of levetiracetam to the levetiracetam binding site.
20
6. A method of discovering or modeling an interaction between an SV2 protein and a compound or agent selected from the group consisting of: levetiracetam, an analog or derivative of levetiracetam, or a compound or agent which competes with levetiracetam or an analog or derivative thereof for binding to the levetiracetam binding site comprising:
25
 - a) contacting the SV2 protein with the compound or agent; and
 - b) measuring and analyzing the interaction of the SV2 protein with the compound or agent.
- 30 7. A method of claim 6 where the analysis is by proteolytic treatment of the SV2 proteins to observe a differential effect of binding of a ligand on proteolytic degradation.

8. A method of claim 6, wherein the analysis is by 3-dimensional modeling or other purely computational techniques.

5 9. A method of claim 8, wherein the 3-dimensional modeling is via nuclear magnetic resonance spectroscopy or X-ray crystallography.

10. A method of claim 6, wherein the analysis is by binding studies.

10 11. A method of any one of claims 6 or 10, wherein the SV2 protein is purified from natural sources

12. A method of claim 11 where the SV2 protein is purified from heterologously expressed protein driven from a cloned nucleotide inserted in an expression vector, in a
15 eukaryotic or prokaryotic host.

13. A method of identifying a levetiracetam binding site within an SV2 protein comprising;

- 20 a) contacting a SV2 protein or fragment thereof with a compound or agent selected from the group consisting of levetiracetam, an analog or derivative of levetiracetam, or a compound or agent which competes with levetiracetam or an analog or derivative thereof for binding to the levetiracetam binding site; and
- 25 b) determining the binding of the compound or agent with the SV2 protein or fragment thereof.

14. A method of claim 13, wherein the SV2 protein or fragment thereof comprises at least one amino acid substitution, deletion or addition.

30 15. A method of claim 14, wherein the addition, deletion or substitution of amino acid residues removes at least one glycosylation sites.

16. A method of claim 15, wherein the removal of glycosylation sites is *via* site-directed mutagenesis.

5 17. A method of claim 13, wherein the SV2 protein is a fusion protein comprising at least one SV2 protein or fragment thereof and a fusion partner.

18. A method of claim 17, wherein the fusion partner is a fusion tag.

10 19. A method of claim 18, wherein the fusion tag is a poly-His tag or glutathione-S-transferase.

20. A method of assaying the interaction between SV2 protein and a second protein comprising;

- 15 a) expressing SV2 protein and the protein of interest in a cell;
 b) exposing the cell to a compound or agent which binds to the levetiracetam binding site; and
 c) determining the interaction between the SV2 protein and the protein of interest.

20

21 A method of claim 20, wherein the second protein is selected from the group consisting of: a cell membrane protein, a vesicle membrane protein, a cytoplasmic protein, a cytoskeletal protein, and an intracellular matrix protein.

25 22. A method of claim 20, wherein the protein of interest is synaptotagmin.

23. A method of claim 20, wherein the protein of interest is a member of the SNARE complex.

30 24. A method of claim 23, wherein the member of the SNARE complex is synaptic vesicle associated VAMP/synaptobrevin, syntaxin, or SNAP-25.

25. A method of claim 20, wherein the SV2 protein lacks at least one glycosylation site.
26. A method of identifying a compound or agent that modulates a neurological disorder
5 associated with synaptic function, endocrinopathy or hormonal disease comprising;
- a) exposing a SV2 protein to the compound or agent; and
 - b) determining whether the compound or agent modulates an activity of the SV2 protein.
- 10 27. A method of any one of claim 3, 4, 5, 6, 20, or 26, wherein the compound or agent is levetiracetam or an analog or derivative thereof, or an anti-SV2 antibody or fragment thereof.
28. A method of any one of claims 13 or 27, wherein the compound or agent competes
15 with levetiracetam or an analog or derivative thereof for binding to the levetiracetam binding site.
29. A method of any one of claims 13 or 27, wherein the compound or agent is an anti-SV2 antibody or fragment thereof.
- 20 30. A method of claim 29, wherein the anti-SV2 antibody or fragment thereof binds to the levetiracetam binding site of SV2 protein.
31. A method of claim 29, wherein the anti-SV2 antibody or fragment thereof is selected
25 from the group consisting of a polyclonal antibody and a monoclonal antibody.
32. A method of claim 31, wherein the antibody fragment is selected from the group consisting of an Fab fragment, Fab' fragment, F(ab')₂ fragment and an scFv fragment.
- 30 33. A method of claim 31, wherein the monoclonal antibody is selected from the group consisting of a chimeric antibody, a humanized antibody, and a human antibody.

34. A method of identifying a cellular response to a compound or agent selected from the group consisting of levetiracetam, an analog or derivative of levetiracetam, or a compound or agent which competes with levetiracetam or an analog or derivative thereof for binding to the levetiracetam binding site comprising:

- a) exposing cells expressing an SV2 protein to the compound or agent; and
- b) analyzing a change in the expression of a nucleic acid or protein in the exposed cell.

35. A method of any one of claims 20 or 34, wherein the step of exposing the cell to a compound or agent which binds to the levetiracetam binding site is carried out under conditions with a divalent cation concentration selected from the group consisting of less than about 1 μM , between about 1 μM and about 1000 μM , and at least about 1000 μM .

36. An isolated nucleic acid molecule comprising the nucleic acid sequence of SEQ ID NO: 5 or the complement thereof.

37. An isolated polypeptide comprising an amino acid sequence encoded by the isolated nucleic acid molecule of claim 123.

38. An isolated polypeptide of claim 124, comprising the amino acid sequence of SEQ ID NO: 6.

39. A method of claim 26, wherein the step of determining whether the compound or agent modulates an activity of the SV2 protein is selected from the group consisting of

a) measuring transport of at least one monovalent cation or divalent cation across a membrane;

b) measuring SNARE complex formation;

c) measuring Ca^{2+} channel formation or activity;

d) measuring SV2 interaction with at least one other protein;

- e) measuring transport of at least one substrate across a membrane; and,
- f) measuring synaptic vesicle fusion, exocytosis, or synaptic vesicle recycling.

40. A method of claim 39, wherein the monovalent cation is selected from the group
5 consisting of H^+ , Cl^- , Na^+ and K^+ .

41. A method of any one of claim 35 or 39, wherein the divalent cation is selected from the group consisting of Ca^{2+} , Zn^{2+} , Pb^{2+} , Mg^{2+} , Mn^{2+} , Fe^{2+} and Cu^{2+} .

10 42. A method of claim 41, wherein the at least one divalent cation is Ca^{2+} .

43. A method of claim 39, wherein the at least one other protein is synaptotagmin.

44. A method of claim 39, wherein the at least one other protein is laminin-1.

15

45. A method of claim 39 wherein the at least one substrate is selected from the group consisting of amines, acetylcholine, excitatory neurotransmitters, GABA, serotonin, glycine or other amino acids, sugars and organic ions.

20 46. A method of identifying a binding partner for a SV2 protein, comprising:
a) exposing a SV2 protein or fragment to a potential binding partner;
b) incubating the protein or fragment and potential binding partner with (2S)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide; and
c) determining if the binding of (2S)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide to the protein is inhibited by the potential binding partner,
25 thereby identifying binding partner for the protein.

47. A method of identifying a compound or agent useful for the treatment of a neurological or endocrinological disorder, comprising:

30 a) exposing a SV2 protein or fragment to the agent and levetiracetam or an analog or derivative thereof; and

b) determining if the binding of levetiracetam or an analog or derivative thereof to the protein is modulated by the agent, thereby identifying an agent useful for the treatment of a neurological disorder.

5 48. A method of claim 47, wherein the levetiracetam or an analog or derivative thereof is directly or indirectly labeled.

49. A method of claim 47, wherein the SV2 protein or fragment is incubated with the levetiracetam or an analog or derivative prior to the agent, after addition of the agent, or
10 concurrent with the agent.

50. A method of claim 47, wherein the SV2 protein or fragment is incubated with levetiracetam.

15 51. A method of claim 47, wherein the neurological disorder is selected from the group consisting of epilepsy; epileptogenesis; seizure disorders; convulsions; withdrawal seizures; neurological disorders; bipolar disorders; mania; depression; anxiety; migraine; neuralgia; trigeminal neuralgia; chronic pain conditions; neuropathic pain; anaesthesia-related hyperexcitability; cerebral ischemia; head trauma; myotonia; excitatory states provoked by
20 drug or alcohol abuse, dependence or withdrawal; stroke; myoclonus; essential tremor; tics; Tourette's syndrome; dyskinesia; spasticity; movement disorders; neonatal cerebral haemorrhage; amyotrophic lateral sclerosis; Parkinson's disease; Alzheimer's disease; a neurodegenerative disease; and dementia.

25 52. A pharmaceutical composition comprising a compound or agent as identified in the method of any one of claims 26 or 47 said compound being different from a compound as described in Fig. 15.

53. A method of treating a neurological or endocrinological disorder which comprises
30 administering to an individual in need of such treatment a compound or agent as identified in the method of any one of claims 26 or 47 said compound being different from a compound as

described in Fig. 15.

54. A method according to claim 53, wherein the neurological disorder is selected from the group consisting of epilepsy; epileptogenesis; seizure disorders; convulsions; withdrawal
5 seizures; neurological disorders; bipolar disorders; mania; depression; anxiety; migraine; neuralgia; trigeminal neuralgia; chronic pain conditions; neuropathic pain; anaesthesia-related hyperexcitability; cerebral ischemia; head trauma; myotonia; excitatory states provoked by drug or alcohol abuse, dependence or withdrawal; stroke; myoclonus; essential tremor; tics; Tourette's syndrome; dyskinesia; spasticity; movement disorders; neonatal cerebral
10 haemorrhage; amyotrophic lateral sclerosis; Parkinson's disease; Alzheimer's disease; a neurodegenerative disease; and dementia.

55. A method according to claim 53 wherein the endocrinological disorders is selected from the group consisting of endocrinopathies involving hypersecretion or hyposecretion of
15 at least one hormone; gigantism; dwarfism; adrenal-medulla-related diseases; hypoglycemia; and circulation shock.

56. A method of any one of claims 1, 20, 26, 34, or 47, wherein the SV2 protein is SV2A.

20 57. A method of claim 56, wherein the SV2A protein comprises SEQ ID NO: 2.

58. A method of claim any one of claims 13, 27, or 47, wherein the analog or derivative of levetiracetam is (2S)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide.

25 59. A method of claim 58, wherein the analog or derivative of levetiracetam is selected from the group consisting of N-alkylated 2-oxo-pyrrolidine derivatives, N-alkylated 2-oxo-piperidinyl derivatives, and N-alkylated 2-oxo-azepanyl derivatives.

60. A method of identifying an agent useful for the treatment of a neurological or
30 endocrinological disorder, comprising:

a) exposing a SV2 protein or fragment to the agent;

- b) incubating the protein or fragment and agent with (2S)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide; and
- c) determining if the binding of (2S)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide to the protein is inhibited by the agent, thereby identifying binding partners for the protein.

61. A method of discovering or modeling an interaction between an SV2 protein, or fragment or derivative thereof, and a compound or agent selected from the group consisting of: levetiracetam, an analog or derivative of levetiracetam, or a compound or agent which competes with levetiracetam or an analog or derivative thereof for binding to the levetiracetam binding site comprising:

- a) creating a 3-dimensional model of the SV2 protein, or fragments thereof, via either biochemical, biophysical, purely computational techniques, or some combination of these; and
- b) creating 3-dimensional model of one or a collection of potential ligands that might potentially bind the SV2 protein.

62. A method of claim 61, further comprising using purely computational techniques to dock the 3-dimensional model of SV2 proteins with the 3-dimensional models of potential ligands.

63. A method of discovering or modeling an interaction between an SV2 protein and a compound or agent selected from the group consisting of: levetiracetam, an analog or derivative of levetiracetam, or a compound or agent which competes with levetiracetam or an analog or derivative thereof for binding to the levetiracetam binding site comprising:

- a) determining a biochemical, pharmacological, organismal, cellular or molecular effect of a potential CNS active molecule in a genetically wild-type animal or in molecules, cells or tissues derived from such animals; and
- b) comparing the measured effect of that compound in an equivalent study in a system with an SV2 protein knocked out or knocked down.

64. A method of isolating a functionally active membrane associated SV2 protein complex comprising:

- a) solubilizing tissues comprising the SV2 protein with a detergent; and
- b) isolating the SV2 protein complex.

5

65. A method of claim 64, wherein the method further comprises purifying the SV2 protein complex by immunoaffinity.

10 66. A method of claim 65, wherein the SV2 protein complex is further purified to obtain the SV2 protein.

67. A method of claim 64, wherein the detergent is n-dodecyl- β -D-maltoside or derivatives or analogs thereof.

15 68. A method of claim 64, wherein the tissues are brain membranes.

69. A method of claim 64, further comprising identifying the molecule or molecules complexed to the SV2 protein.

20 70. A method of any one of claims 64 to 69, wherein the SV2 protein is SV2A protein, SV2B protein, or SV2C protein.

71. A purified SV2 protein complex obtained by the method of claim 64.

25 72. A purified SV2 protein complex of claim 71, wherein the SV2 protein is SV2A protein, SV2B protein, or SV2C protein.

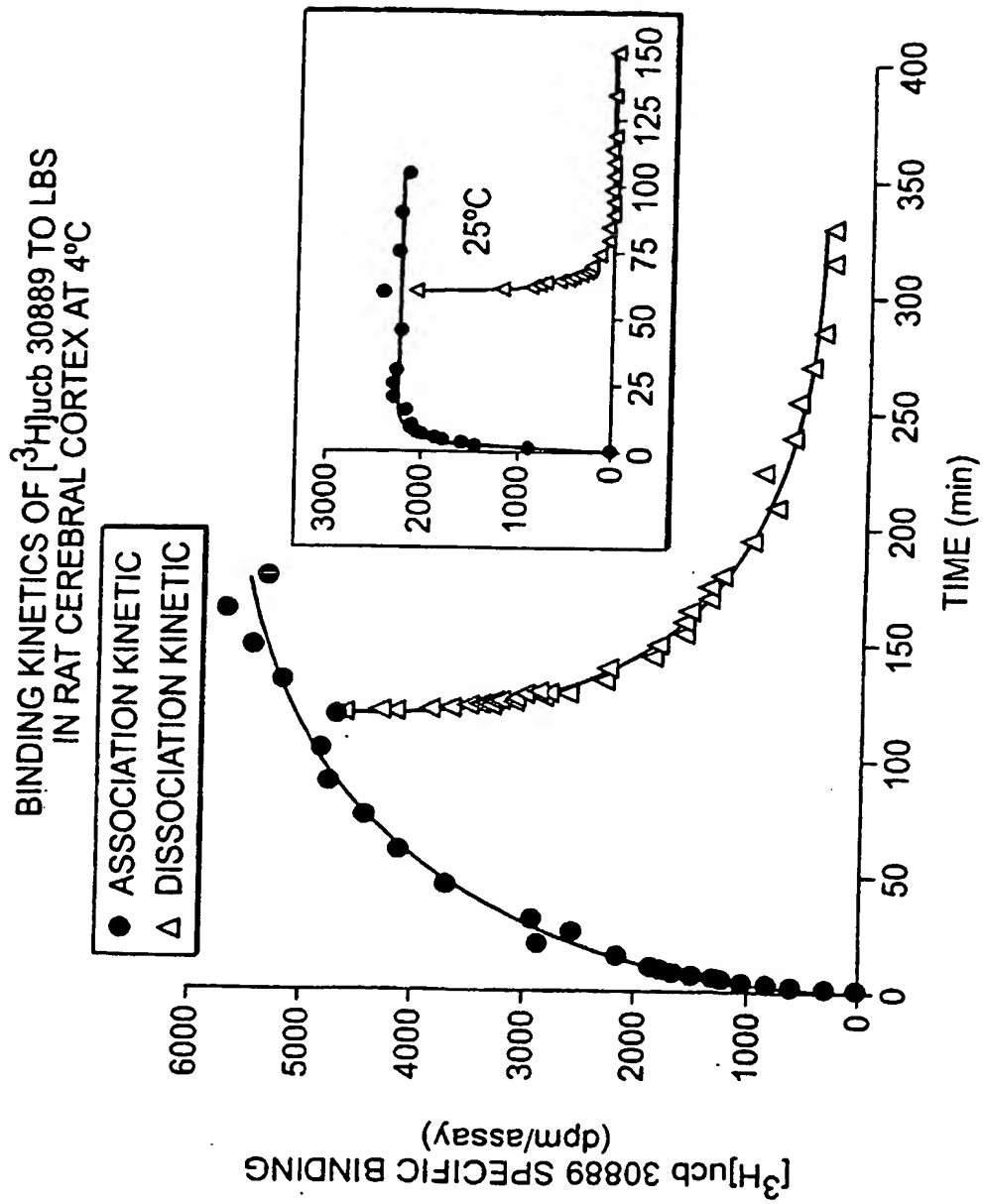


FIG. 1

$[^3\text{H}]\text{jucb 30889}$ SATURATION BINDING CURVE
- RAT CEREBRAL CORTEX -

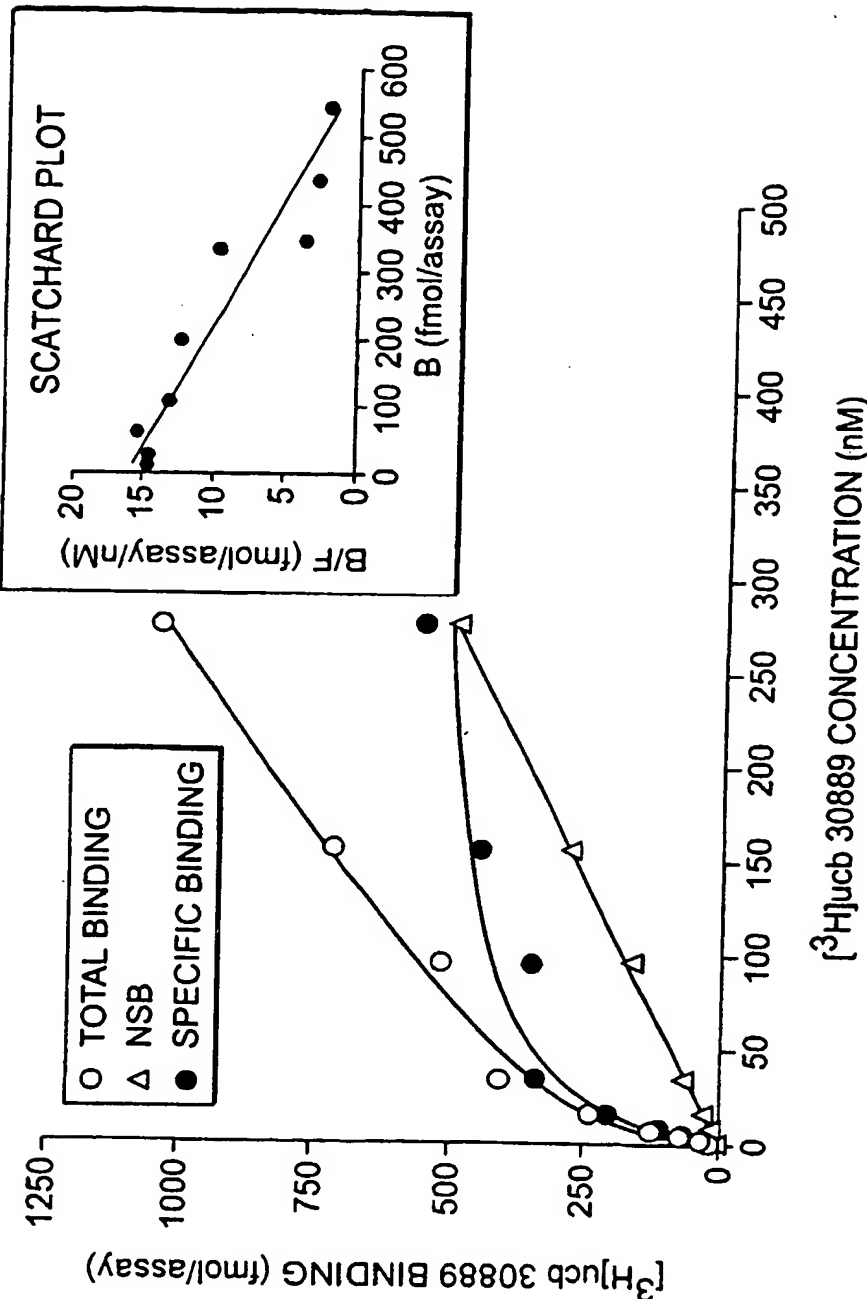


FIG. 2

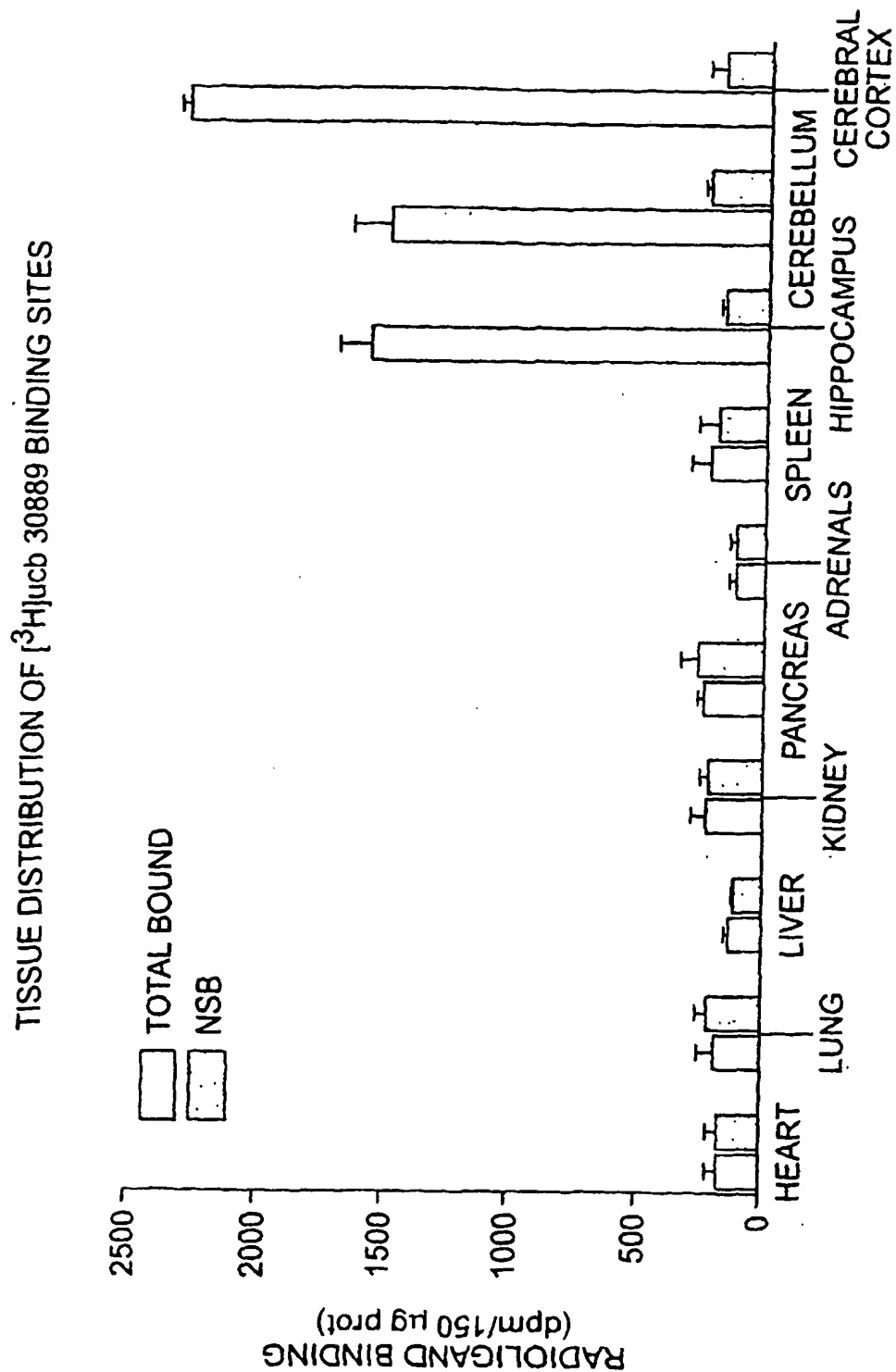
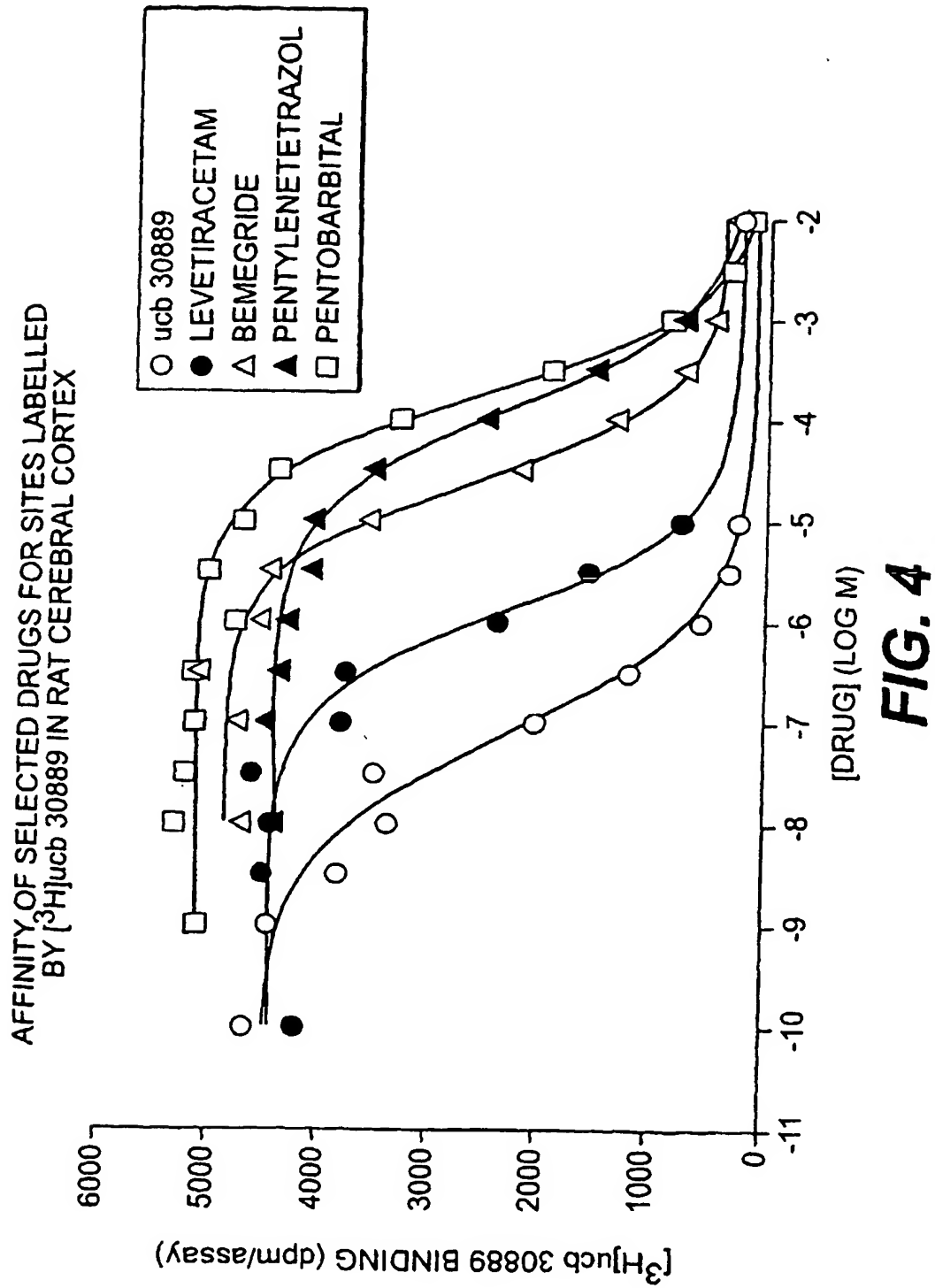
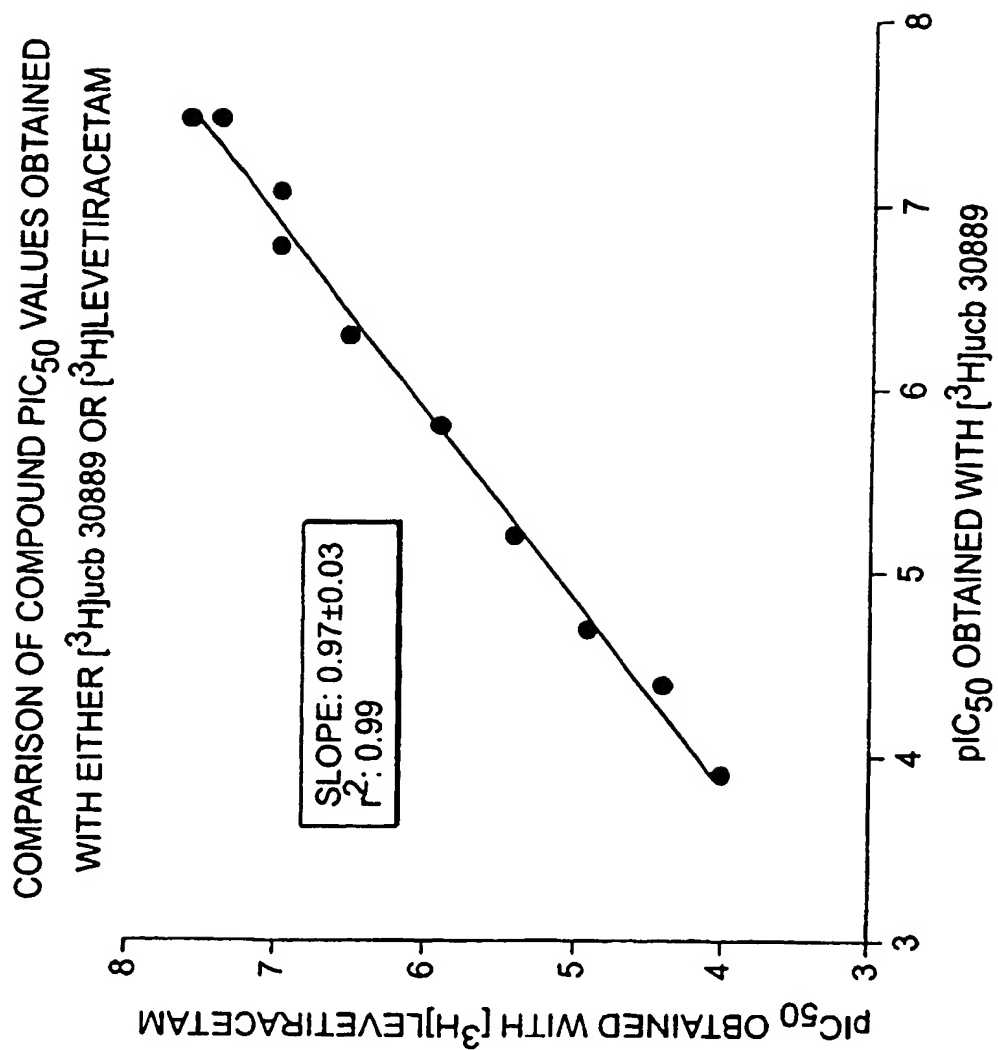


FIG. 3



**FIG. 5**

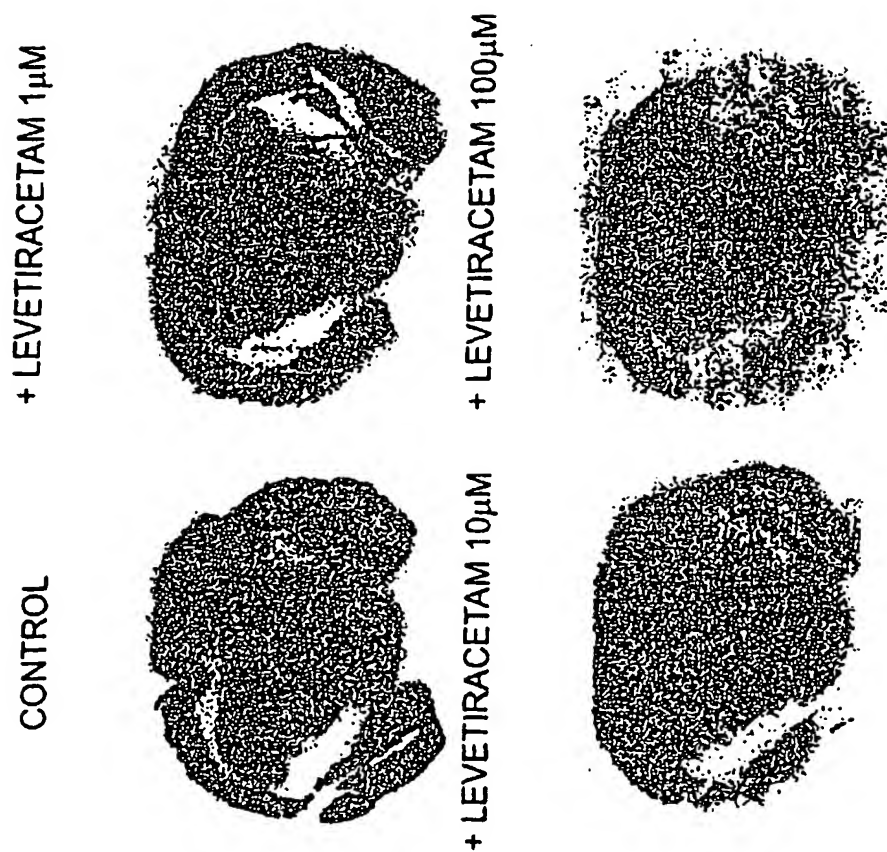


FIG. 6



FIG. 7a



FIG. 7b



FIG. 7c



FIG. 7d

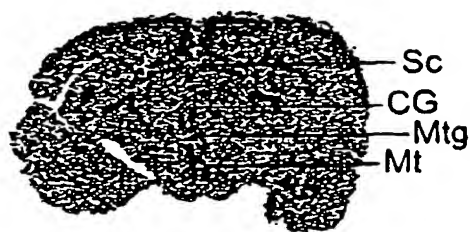


FIG. 7e



FIG. 7f

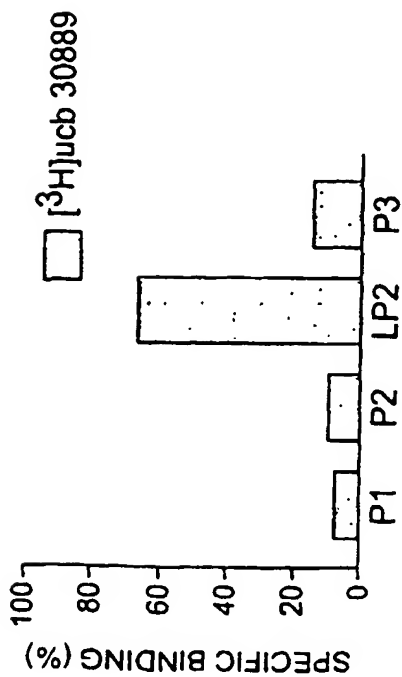


FIG. 8a

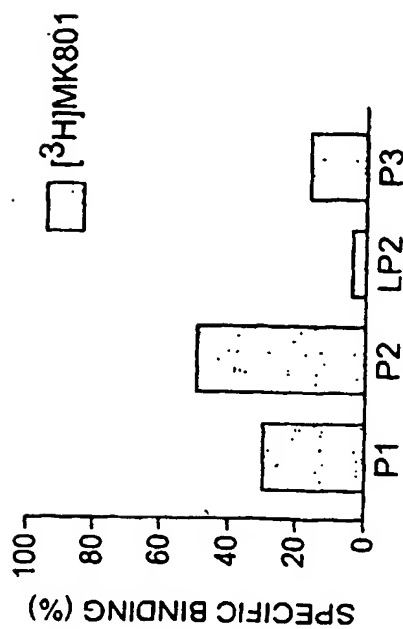


FIG. 8c

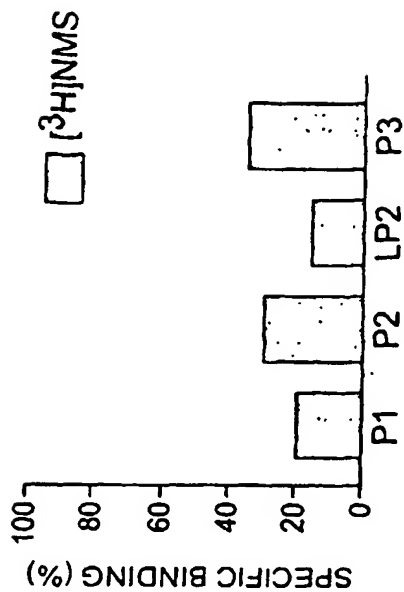


FIG. 8b

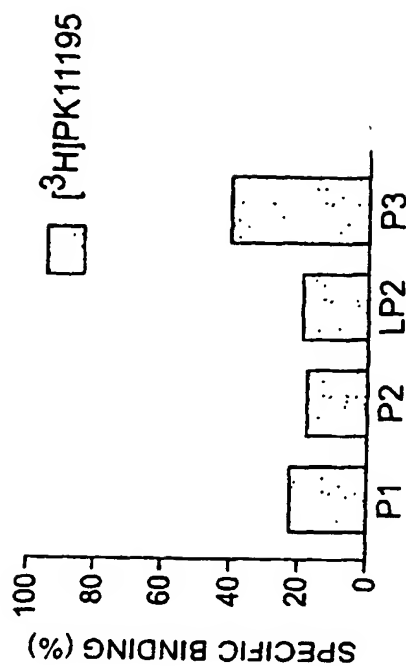


FIG. 8d

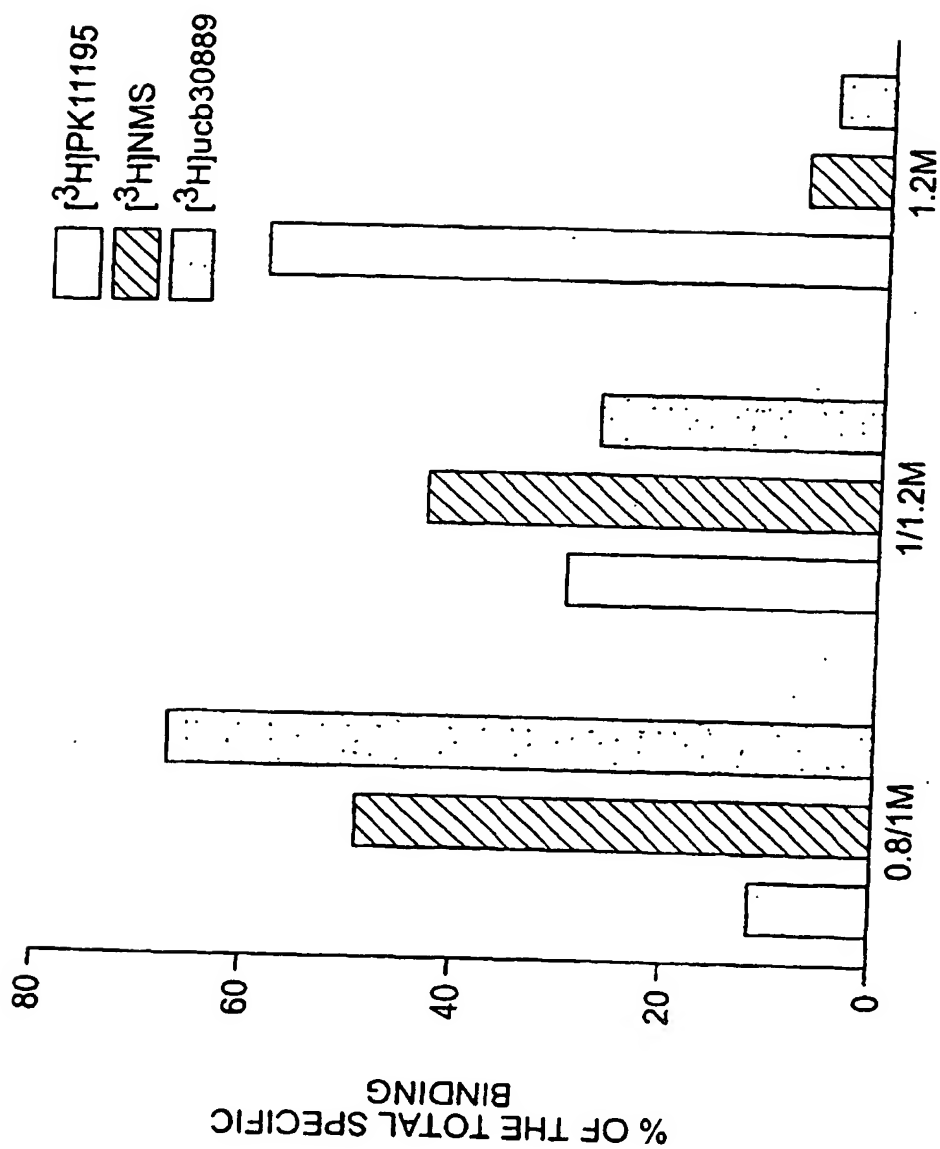


FIG. 9

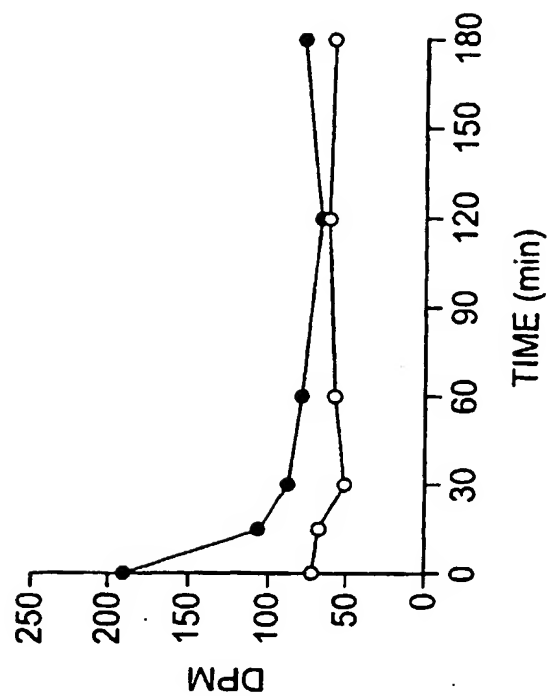


FIG. 10a

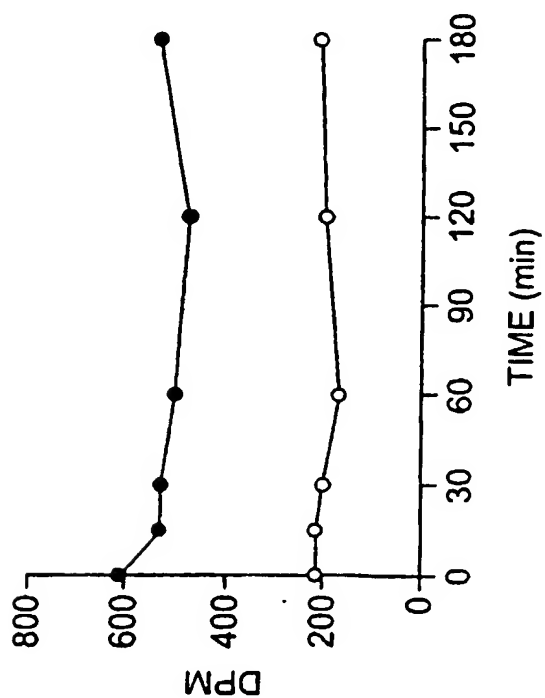
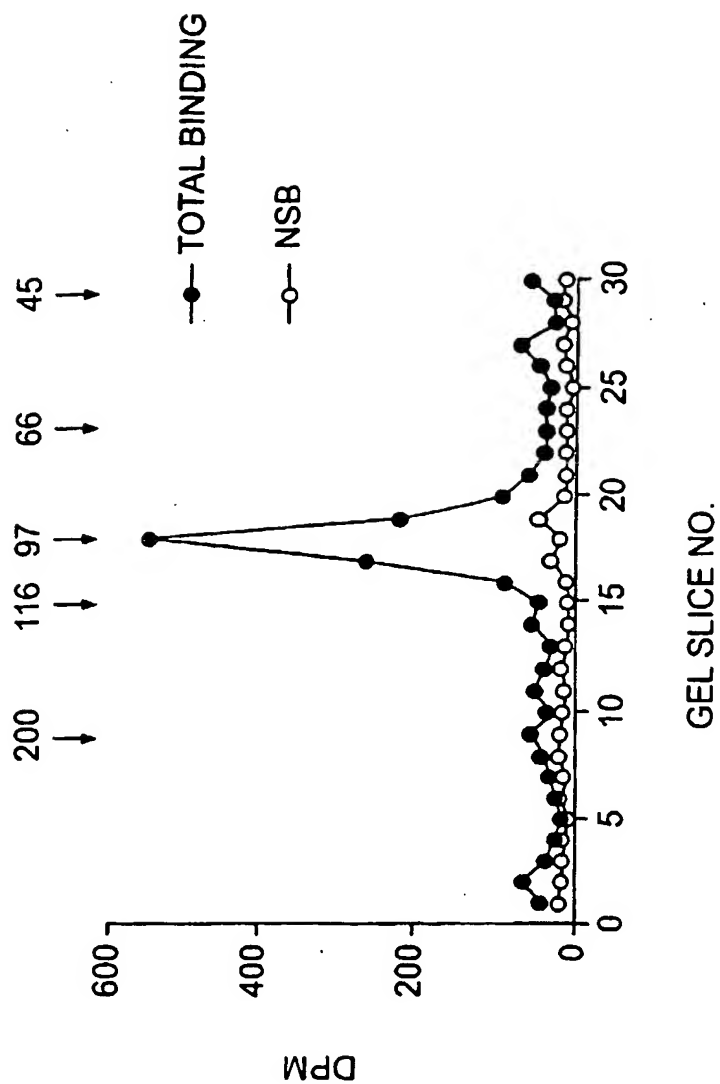


FIG. 10b

**FIG. 11**

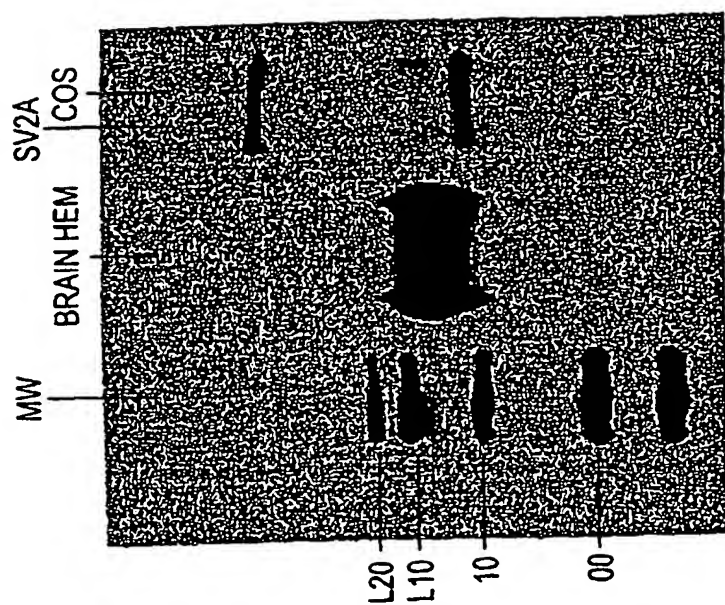
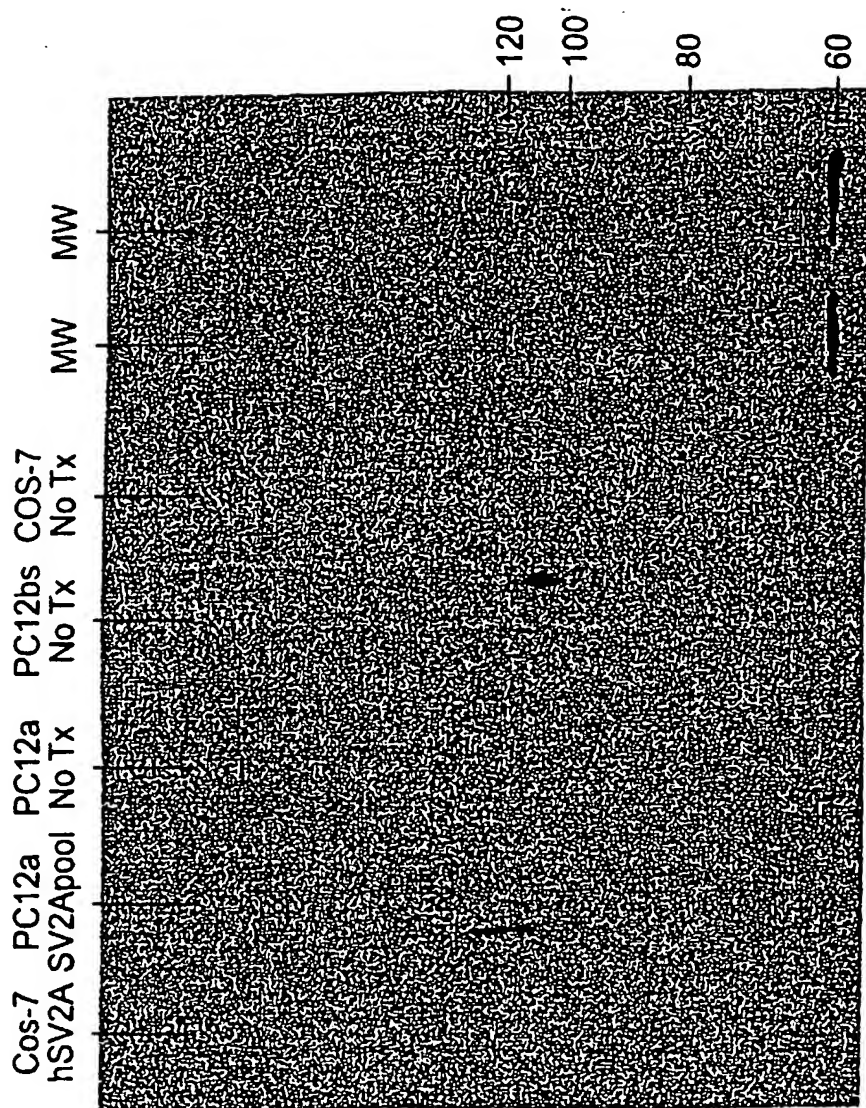


FIG. 12a

**FIG. 12b**

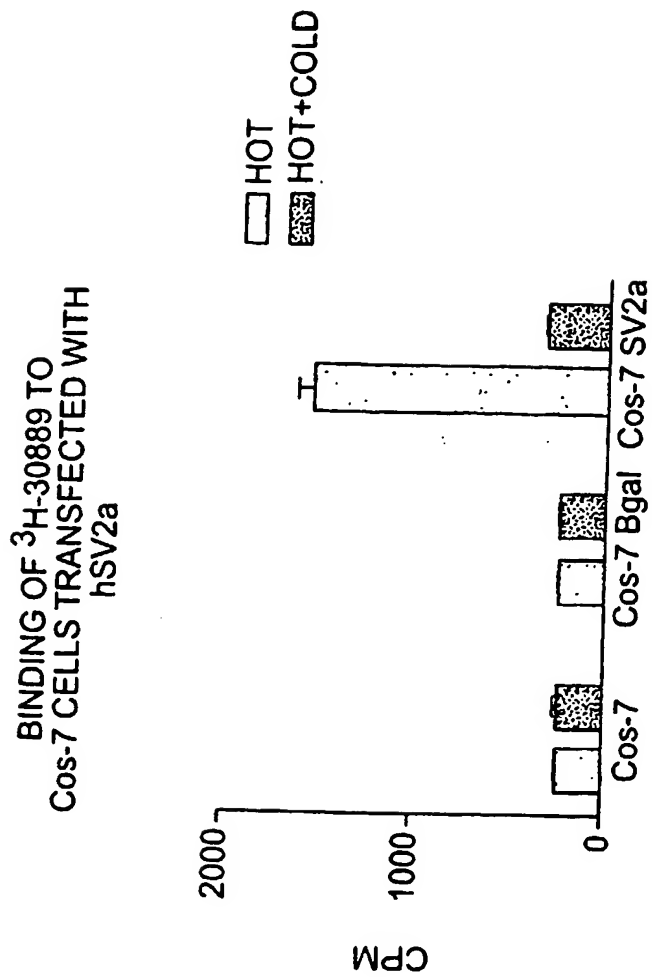
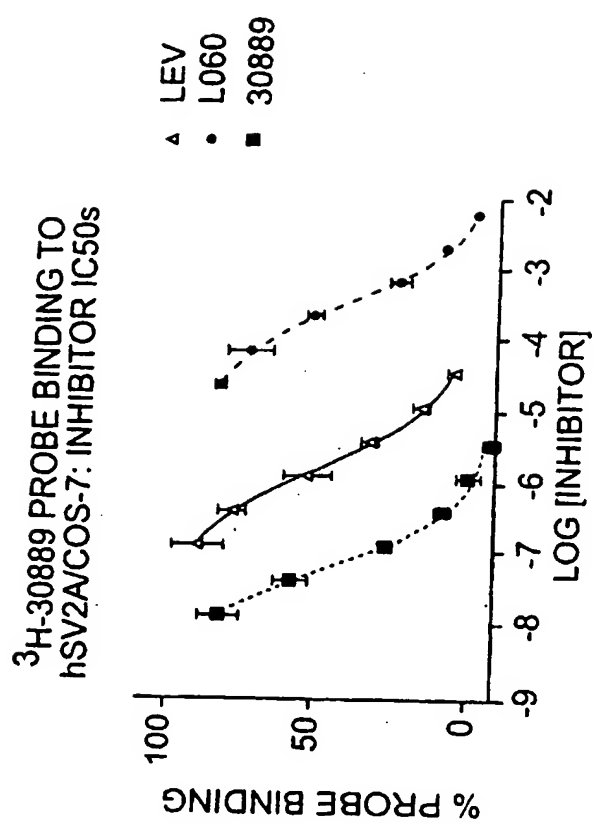


FIG. 13

**FIG. 14**

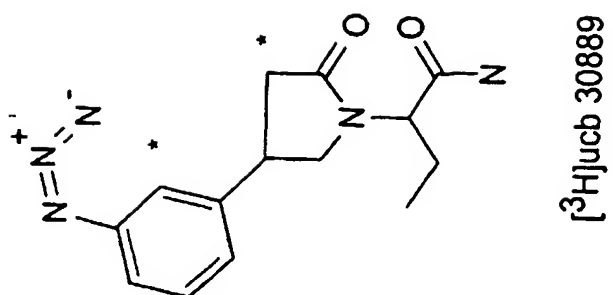


FIG. 15b

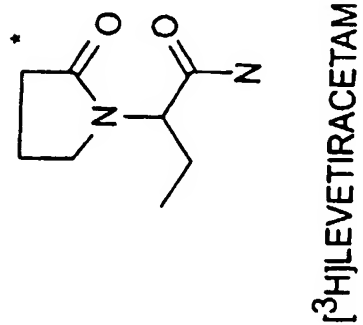


FIG. 15a

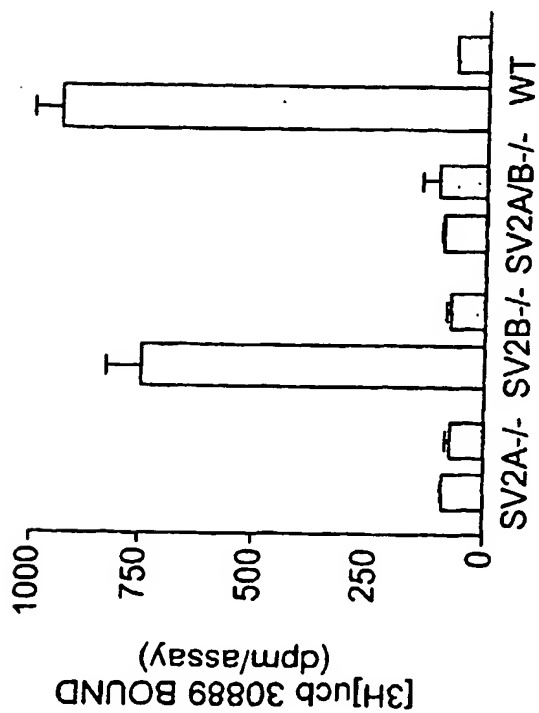


FIG. 16a

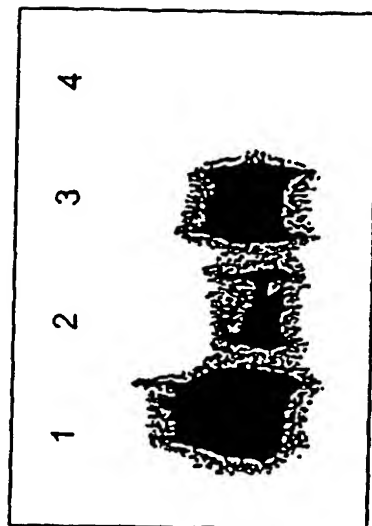


FIG. 16b

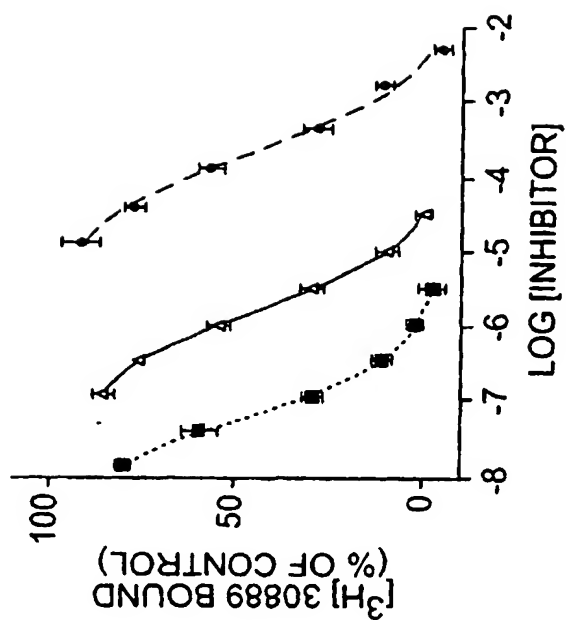


FIG. 17b

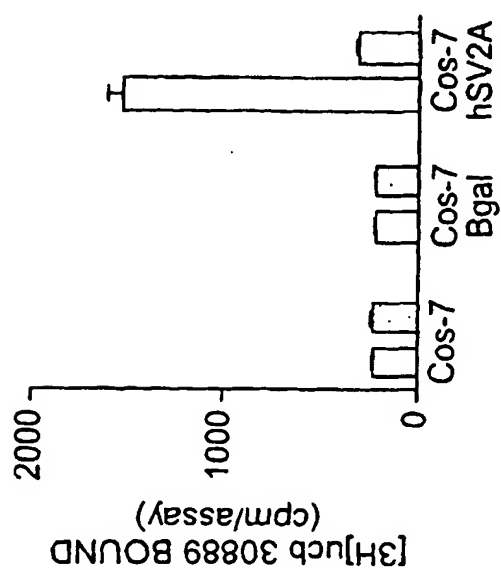
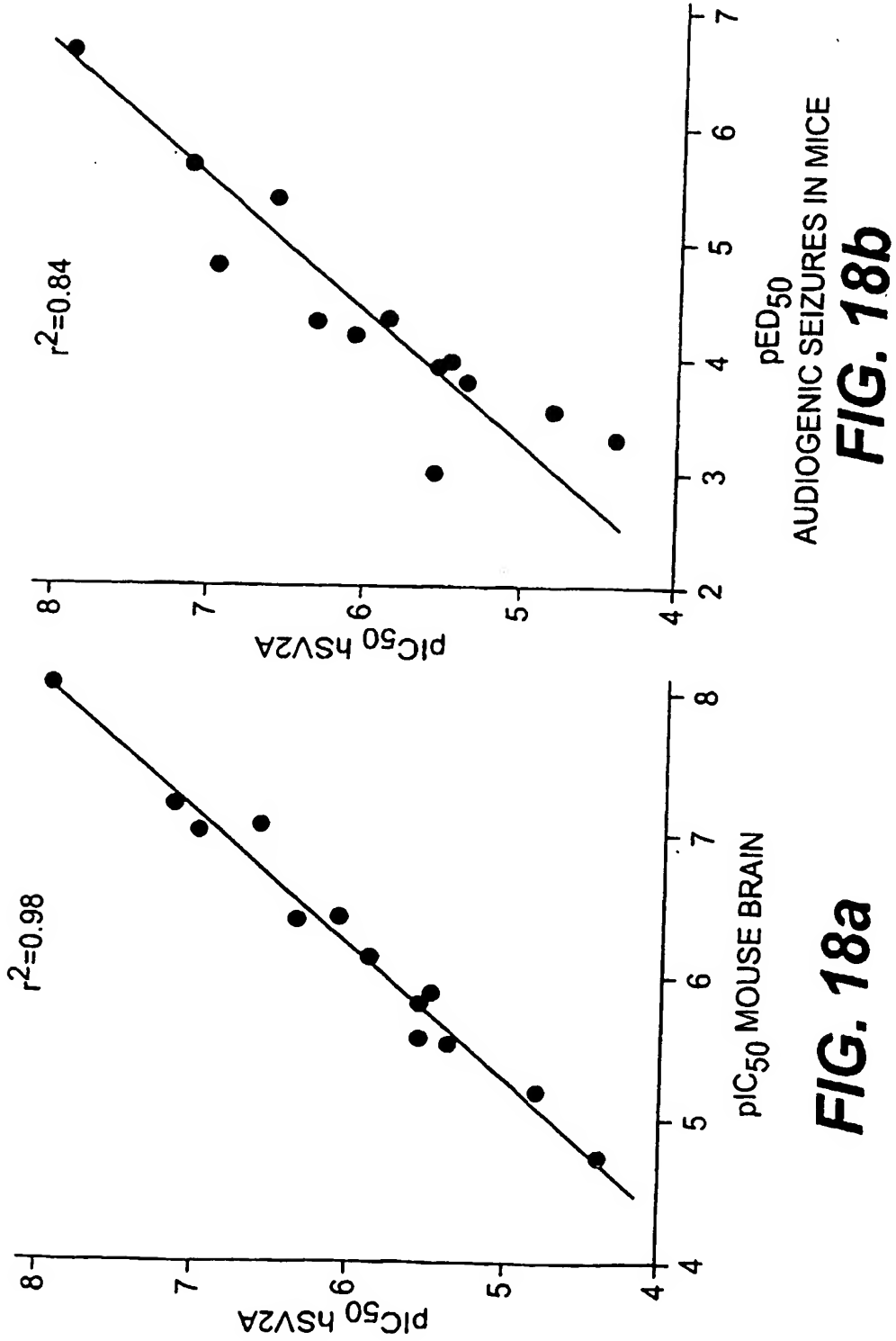
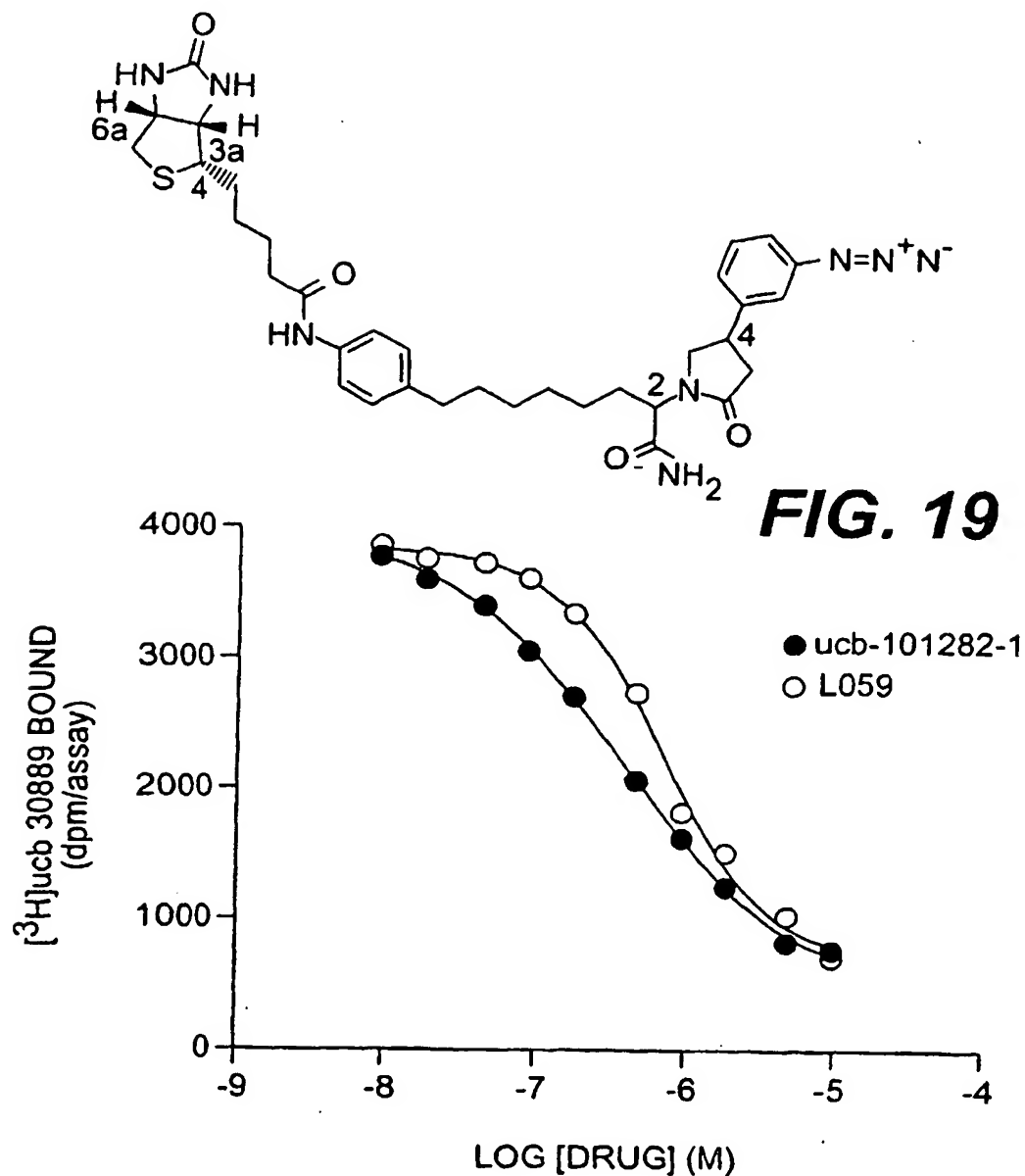


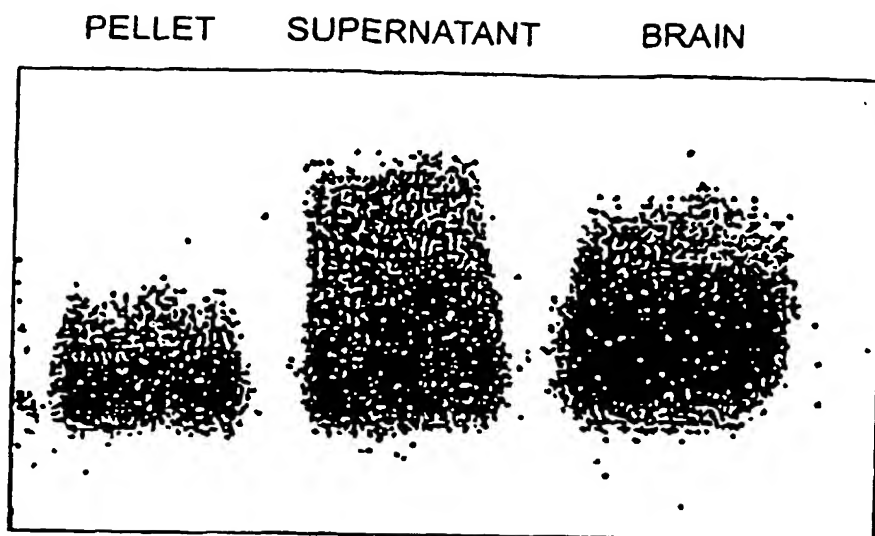
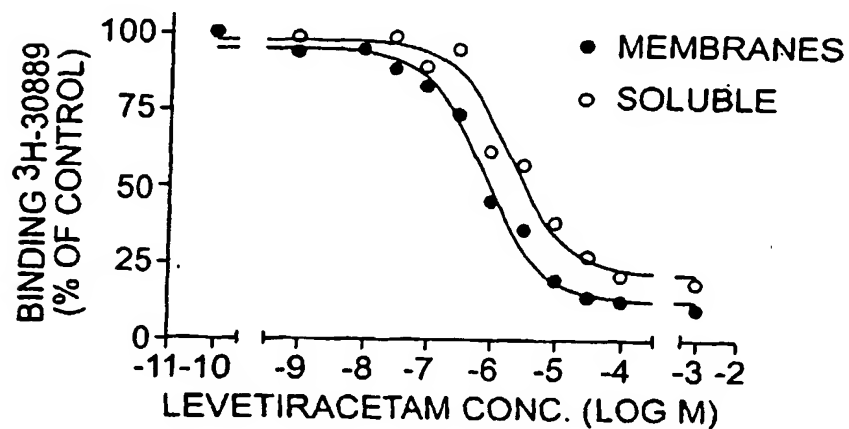
FIG. 17a





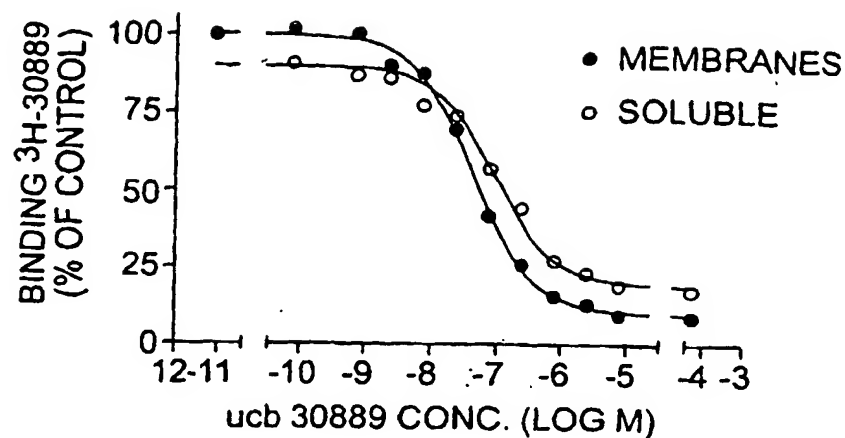
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BOTTOM	458.4	661.8
TOP	3923	3836
LOGEC50	-6.392	-6.129
HILLSLOPE	-0.7946	-1.237
EC50	4.0510e-007	7.4320e-007

FIG. 20

**FIG. 21a**

	MEMBRANES	SOLUBLE EXTRACTS
BOTTOM	94.97	97.71
TOP	12.30	21.03
LEGE50	-6.104	-5.692
EC50	7.8770e-007	2.0340e-006

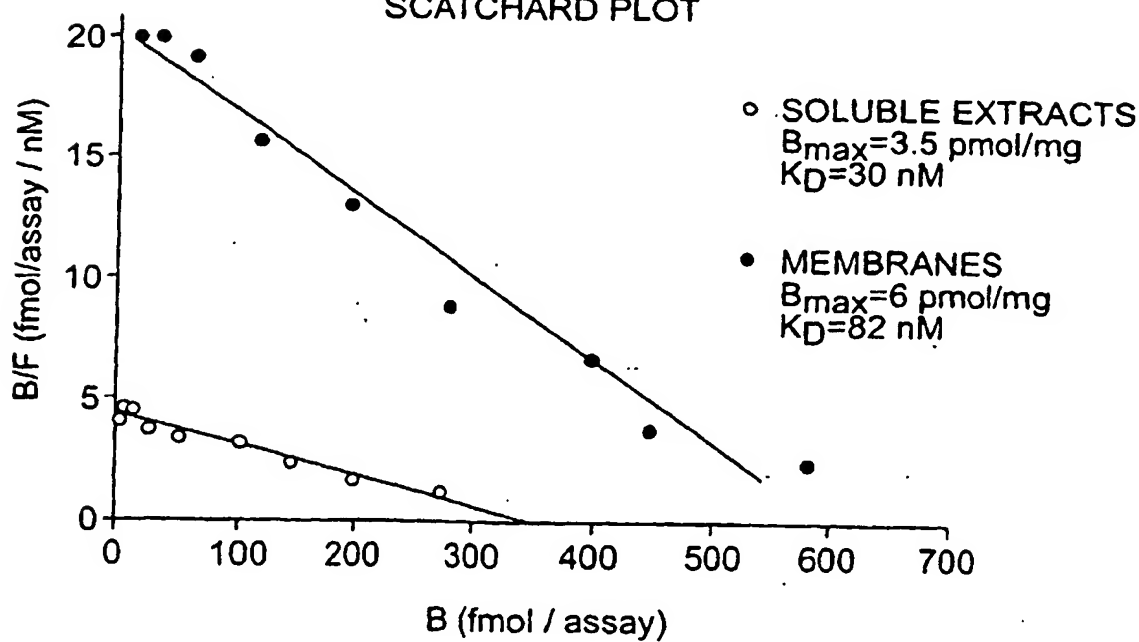
FIG. 21b



	MEMBRANES	SOLUBLE EXTRACTS
BOTTOM	100.7	90.62
TOP	10.63	19.87
LEGE _{EC50}	-7.235	-6.922
EC ₅₀	5.8200e-008	1.1970e-007

FIG. 21c

SCATCHARD PLOT

**FIG. 22a**

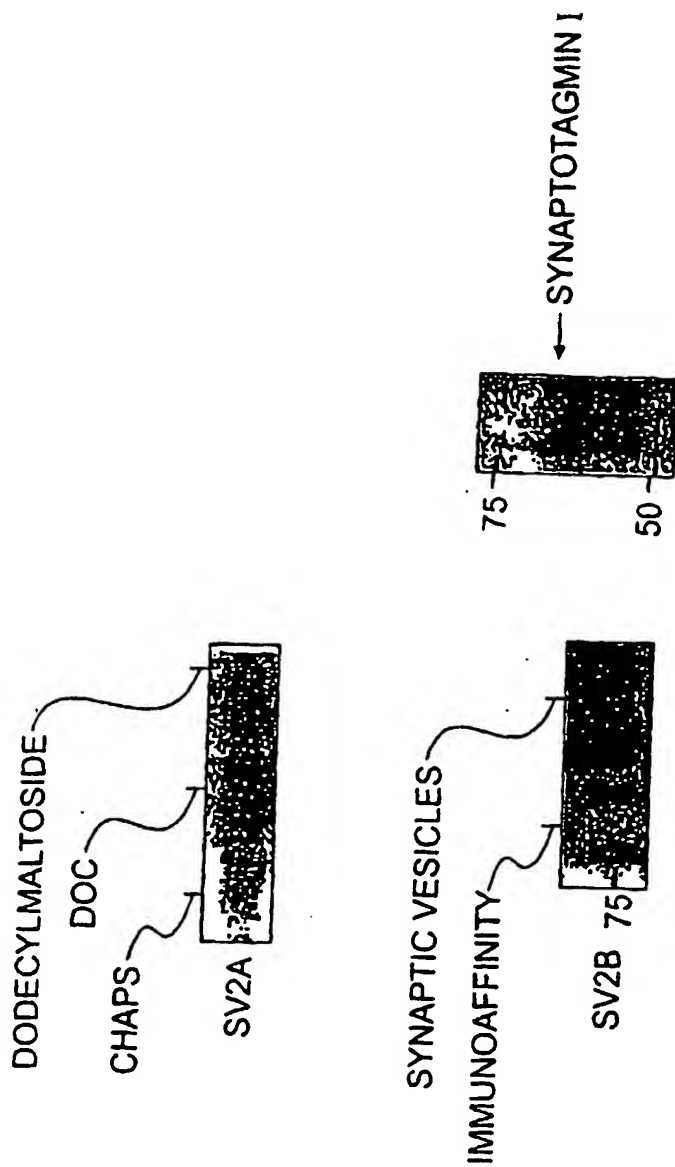


FIG. 22b

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 LYNCH, Berkley
 NOCKA, Karl
 FUKS, Bruno

<120> Methods for the identification of agents for the treatment of seizures, neurological diseases, endocrinopathies and hormonal diseases

<130> 53529-5007-01-WO

<150> US 60/430,372
 <151> 2002-12-03

<150> US 60/506,764
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gac att gct aag gaa gtc aaa aag cat gcg gcc aag aag gtg gtg aag	96
Asp Ile Ala Lys Glu Val Lys Lys His Ala Ala Lys Lys Val Val Lys	
20 25 30	
ggc ctg gac aga gtc cag gac gaa tat tcc cga aga tcg tac tcc cgc	144
Gly Leu Asp Arg Val Gln Asp Glu Tyr Ser Arg Arg Ser Tyr Ser Arg	
35 40 45	
ttt gag gag gag gat gat gat gat gac ttc cct gct ccc agt gat ggt	192
Phe Glu Glu Glu Asp Asp Asp Asp Asp Phe Pro Ala Pro Ser Asp Gly	
50 55 60	
tat tac cga gga gaa ggg acc cag gat gag gag gaa ggt ggt gca tcc	240
Tyr Tyr Arg Gly Glu Gly Thr Gln Asp Glu Glu Glu Gly Gly Ala Ser	
65 70 75 80	
agt gat gct act gag ggc cat gac gag gat gat gag atc tat gaa ggg	288
Ser Asp Ala Thr Glu Gly His Asp Glu Asp Asp Glu Ile Tyr Glu Gly	
85 90 95	
gaa tat cag ggc att ccc cgg gca gag tct ggg ggc aaa ggc gag cgg	336
Glu Tyr Gln Gly Ile Pro Arg Ala Glu Ser Gly Gly Lys Gly Glu Arg	
100 105 110	

acg cag cct gag agc ccc cgt ttc ttc cta gag aat gga aag cat gat Thr Gln Pro Glu Ser Pro Arg Phe Phe Leu Glu Asn Gly Lys His Asp 355 360 365	1104
gag gcc tgg atg gtg ctg aag cag gtc cat gat acc aac atg cga gcc Glu Ala Trp Met Val Leu Lys Gln Val His Asp Thr Asn Met Arg Ala 370 375 380	1152
aaa gga cat cct gag cga gtg ttc tca gta acc cac att aag acg att Lys Gly His Pro Glu Arg Val Phe Ser Val Thr His Ile Lys Thr Ile 385 390 395 400	1200
cat cag gag gat gaa ttg att gag atc cag tcg gac aca ggg acc tgg His Gln Glu Asp Glu Leu Ile Glu Ile Gln Ser Asp Thr Gly Thr Trp 405 410 415	1248
tac cag cgc tgg ggg gtc cgg gcc ttg agc cta ggg ggg cag gtt tgg Tyr Gln Arg Trp Gly Val Arg Ala Leu Ser Leu Gly Gly Gln Val Trp 420 425 430	1296
ggg aat ttt ctc tcc tgt ttt ggt ccc gaa tat cgg cgc atc act ctg Gly Asn Phe Leu Ser Cys Phe Gly Pro Glu Tyr Arg Arg Ile Thr Leu 435 440 445	1344
atg atg atg ggt gtg tgg ttc acc atg tca ttc agc tac tat ggc ctg Met Met Met Gly Val Trp Phe Thr Met Ser Phe Ser Tyr Tyr Gly Leu 450 455 460	1392
acc gtc tgg ttt cct gac atg atc cgc cat ctc cag gca gtg gac tac Thr Val Trp Phe Pro Asp Met Ile Arg His Leu Gln Ala Val Asp Tyr 465 470 475 480	1440
gca tcc cgc acc aaa gtg ttc ccc ggg gag cgc gta gag cat gta act Ala Ser Arg Thr Lys Val Phe Pro Gly Glu Arg Val Glu His Val Thr 485 490 495	1488
ttt aac ttc acg ttg gag aat cag atc cac cga ggc ggg cag tac ttc Phe Asn Phe Thr Leu Glu Asn Gln Ile His Arg Gly Gly Gln Tyr Phe 500 505 510	1536
aat gac aag ttc att ggg ctg cgg ctc aag tca gtg tcc ttt gag gat Asn Asp Lys Phe Ile Gly Leu Arg Leu Lys Ser Val Ser Phe Glu Asp 515 520 525	1584
tcc ctg ttt gaa gag tgt tat ttt gag gat gtc aca tcc agc aac acg Ser Leu Phe Glu Glu Cys Tyr Phe Glu Asp Val Thr Ser Ser Asn Thr 530 535 540	1632
ttt ttc cgc aac tgc aca ttc atc aac act gtg ttc tat aac act gac Phe Phe Arg Asn Cys Thr Phe Ile Asn Thr Val Phe Tyr Asn Thr Asp 545 550 555 560	1680
ctg ttc gag tac aag ttt gtg aac agc cgt ctg ata aac agt aca ttc Leu Phe Glu Tyr Lys Phe Val Asn Ser Arg Leu Ile Asn Ser Thr Phe 565 570 575	1728
ctg cac aac aag gag ggc tgc ccg cta gac gtg aca ggg acg ggc gaa Leu His Asn Lys Glu Gly Cys Pro Leu Asp Val Thr Gly Thr Gly Glu 580 585 590	1776
ggg gcc tac atg gta tac ttt gtg agc ttc ctg ggg aca ctg gca gtg	1824

Ser Asp Ala Thr Glu Gly His Asp Glu Asp Asp Glu Ile Tyr Glu Gly
 85 90 95
 Glu Tyr Gln Gly Ile Pro Arg Ala Glu Ser Gly Gly Lys Gly Glu Arg
 100 105 110
 Met Ala Asp Gly Ala Pro Leu Ala Gly Val Arg Gly Gly Leu Ser Asp
 115 120 125
 Gly Glu Gly Pro Pro Gly Gly Arg Gly Glu Ala Gln Arg Arg Lys Glu
 130 135 140
 Arg Glu Glu Leu Ala Gln Gln Tyr Glu Ala Ile Leu Arg Glu Cys Gly
 145 150 155 160
 His Gly Arg Phe Gln Trp Thr Leu Tyr Phe Val Leu Gly Leu Ala Leu
 165 170 175
 Met Ala Asp Gly Val Glu Val Phe Val Val Gly Phe Val Leu Pro Ser
 180 185 190
 Ala Glu Lys Asp Met Cys Leu Ser Asp Ser Asn Lys Gly Met Leu Gly
 195 200 205
 Leu Ile Val Tyr Leu Gly Met Met Val Gly Ala Phe Leu Trp Gly Gly
 210 215 220
 Leu Ala Asp Arg Leu Gly Arg Arg Gln Cys Leu Leu Ile Ser Leu Ser
 225 230 235 240
 Val Asn Ser Val Phe Ala Phe Phe Ser Ser Phe Val Gln Gly Tyr Gly
 245 250 255
 Thr Phe Leu Phe Cys Arg Leu Leu Ser Gly Val Gly Ile Gly Gly Ser
 260 265 270
 Ile Pro Ile Val Phe Ser Tyr Phe Ser Glu Phe Leu Ala Gln Glu Lys
 275 280 285
 Arg Gly Glu His Leu Ser Trp Leu Cys Met Phe Trp Met Ile Gly Gly
 290 295 300
 Val Tyr Ala Ala Ala Met Ala Trp Ala Ile Ile Pro His Tyr Gly Trp
 305 310 315 320
 Ser Phe Gln Met Gly Ser Ala Tyr Gln Phe His Ser Trp Arg Val Phe
 325 330 335
 Val Leu Val Cys Ala Phe Pro Ser Val Phe Ala Ile Gly Ala Leu Thr
 340 345 350
 Thr Gln Pro Glu Ser Pro Arg Phe Phe Leu Glu Asn Gly Lys His Asp
 355 360 365
 Glu Ala Trp Met Val Leu Lys Gln Val His Asp Thr Asn Met Arg Ala
 370 375 380
 Lys Gly His Pro Glu Arg Val Phe Ser Val Thr His Ile Lys Thr Ile
 385 390 395 400
 His Gln Glu Asp Glu Leu Ile Glu Ile Gln Ser Asp Thr Gly Thr Trp

Arg Gly Gln Val Leu Gln
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<213> Homo sapiens

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Asp Gly Tyr Tyr Arg Gly Asn Glu Ser Asn Pro Glu Glu Asp Ala Gln
20 25 30
agt gat gtc acc gaa ggc cat gat gag gaa gac gag atc tat gag ggc 144
Ser Asp Val Thr Glu Gly His Asp Glu Glu Asp Glu Ile Tyr Glu Gly
35 40 45
gag tac cag ggt atc cct cac cca gat gat gtc aag gcc aag cag gcc 192
Glu Tyr Gln Gly Ile Pro His Pro Asp Asp Val Lys Ala Lys Gln Ala
50 55 60
aag atg gcg ccc tcc aga atg gac agc ctt cgg ggc cag aca gac ctg 240
Lys Met Ala Pro Ser Arg Met Asp Ser Leu Arg Gly Gln Thr Asp Leu
65 70 75 80
atg gct gag agg ctg gaa gat gag gag cag ttg gcc cac cag tac gag 288
Met Ala Glu Arg Leu Glu Asp Glu Glu Gln Leu Ala His Gln Tyr Glu
85 90 95
acc atc atg gat gag tgt ggc cat ggc cgc ttc cag tgg atc ctc ttt 336
Thr Ile Met Asp Glu Cys Gly His Gly Arg Phe Gln Trp Ile Leu Phe
100 105 110
ttc gtc ttg ggt ttg gcc ctg atg gcc gat ggg gtg gaa gtg ttc gtg 384
Phe Val Leu Gly Leu Ala Leu Met Ala Asp Gly Val Glu Val Phe Val
115 120 125
gtg agt ttt gcc ctg ccc agt gca gag aag gac atg tgt ctg tcc agt 432
Val Ser Phe Ala Leu Pro Ser Ala Glu Lys Asp Met Cys Leu Ser Ser
130 135 140
tcc aaa aaa gga atg cta ggg atg ata gtc tac ttg gga atg atg gcg 480
Ser Lys Lys Gly Met Leu Gly Met Ile Val Tyr Leu Gly Met Met Ala
145 150 155 160
ggc gcc ttc atc ctg gga ggc ctg gct gat aag ctg gga agg aag cga 528
Gly Ala Phe Ile Leu Gly Gly Leu Ala Asp Lys Leu Gly Arg Lys Arg
165 170 175
gtc ctc agc atg tct ctg gcc gtc aat gcc tcc ttc gcc tcc ctc tct 576

420	425	430	
gag cat gtg tac ggc gcc aca atc aac ttc acg atg gaa aat cag atc Glu His Val Tyr Gly Ala Thr Ile Asn Phe Thr Met Glu Asn Gln Ile 435 440 445			1344
cac caa cat ggg aaa ctt gtg aat gat aag ttc aca aga atg tac ttt His Gln His Gly Lys Leu Val Asn Asp Lys Phe Thr Arg Met Tyr Phe 450 455 460			1392
aaa cat gta ctc ttt gag gac aca ttc ttt gag gag tgc tat ttt gaa Lys His Val Leu Phe Glu Asp Thr Phe Phe Asp Glu Cys Tyr Phe Glu 465 470 475 480			1440
gac gta aca tca aca gat acc tac ttc aaa aat tgt acc att gaa tca Asp Val Thr Ser Thr Asp Thr Tyr Phe Lys Asn Cys Thr Ile Glu Ser 485 490 495			1488
acc atc ttt tac aac aca gac ctc tac gag cac aag ttc atc aac tgt Thr Ile Phe Tyr Asn Thr Asp Leu Tyr Glu His Lys Phe Ile Asn Cys 500 505 510			1536
cgg ttt atc aac tcc acc ttc ctg gag cag aag gag ggc tgc cac atg Arg Phe Ile Asn Ser Thr Phe Leu Glu Gln Lys Glu Gly Cys His Met 515 520 525			1584
gac ttg gag caa gat aat gac ttc ctg att tac ctc gtc agc ttc ctg Asp Leu Glu Gln Asp Asn Asp Phe Leu Ile Tyr Leu Val Ser Phe Leu 530 535 540			1632
ggc agc ctg tct gtc tta ccc ggg aac atc att tct gcc ctg ctc atg Gly Ser Leu Ser Val Leu Pro Gly Asn Ile Ile Ser Ala Leu Leu Met 545 550 555 560			1680
gat aga att gga agg ctc aag atg att ggt ggc tcc atg cta atc tct Asp Arg Ile Gly Arg Leu Lys Met Ile Gly Gly Ser Met Leu Ile Ser 565 570 575			1728
gca gtc tgc tgc ttc ttc ctg ttt ttt ggc aac agt gag tct gca atg Ala Val Cys Cys Phe Phe Leu Phe Phe Gly Asn Ser Glu Ser Ala Met 580 585 590			1776
atc ggc tgg cag tgc ctg ttc tgt ggg aca agc att gca gcc tgg aat Ile Gly Trp Gln Cys Leu Phe Cys Gly Thr Ser Ile Ala Ala Trp Asn 595 600 605			1824
gct ctg gat gtg atc aca gtg gag ctg tat ccc acc aac cag aga gca Ala Leu Asp Val Ile Thr Val Glu Leu Tyr Pro Thr Asn Gln Arg Ala 610 615 620			1872
aca gcc ttc ggc att ctc aat gga tta tgc aaa ttt ggc gcc atc ctg Thr Ala Phe Gly Ile Leu Asn Gly Leu Cys Lys Phe Gly Ala Ile Leu 625 630 635 640			1920
gga aac acc atc ttt gct tct ttt gtt ggg ata acc aaa gtg gtc ccc Gly Asn Thr Ile Phe Ala Ser Phe Val Gly Ile Thr Lys Val Val Pro 645 650 655			1968
atc ctt ctg gct gct gct tct ctg gtt ggg ggt ggc ctg att gcc ctt Ile Leu Leu Ala Ala Ala Ser Leu Val Gly Gly Gly Leu Ile Ala Leu 660 665 670			2016

260	265	270
Phe His Ser Trp Arg Val Phe Val Ile Val Cys Ala Leu Pro Cys Thr		
275	280	285
Val Ser Met Val Ala Leu Lys Phe Met Pro Glu Ser Pro Arg Phe Leu		
290	295	300
Leu Glu Met Gly Lys His Asp Glu Ala Trp Met Ile Leu Lys Gln Val		
305	310	315
His Asp Thr Asn Met Arg Ala Lys Gly Thr Pro Glu Lys Val Phe Thr		
325	330	335
Val Ser Asn Ile Lys Thr Pro Lys Gln Met Asp Glu Phe Ile Glu Ile		
340	345	350
Gln Ser Ser Thr Gly Thr Trp Tyr Gln Arg Trp Leu Val Arg Phe Lys		
355	360	365
Thr Ile Phe Lys Gln Val Trp Asp Asn Ala Leu Tyr Cys Val Met Gly		
370	375	380
Pro Tyr Arg Met Asn Thr Leu Ile Leu Ala Val Val Trp Phe Ala Met		
385	390	395
Ala Phe Ser Tyr Tyr Gly Leu Thr Val Trp Phe Pro Asp Met Ile Arg		
405	410	415
Tyr Phe Gln Asp Glu Glu Tyr Lys Ser Lys Met Lys Val Phe Phe Gly		
420	425	430
Glu His Val Tyr Gly Ala Thr Ile Asn Phe Thr Met Glu Asn Gln Ile		
435	440	445
His Gln His Gly Lys Leu Val Asn Asp Lys Phe Thr Arg Met Tyr Phe		
450	455	460
Lys His Val Leu Phe Glu Asp Thr Phe Phe Asp Glu Cys Tyr Phe Glu		
465	470	475
Asp Val Thr Ser Thr Asp Thr Tyr Phe Lys Asn Cys Thr Ile Glu Ser		
485	490	495
Thr Ile Phe Tyr Asn Thr Asp Leu Tyr Glu His Lys Phe Ile Asn Cys		
500	505	510
Arg Phe Ile Asn Ser Thr Phe Leu Glu Gln Lys Glu Gly Cys His Met		
515	520	525
Asp Leu Glu Gln Asp Asn Asp Phe Leu Ile Tyr Leu Val Ser Phe Leu		
530	535	540
Gly Ser Leu Ser Val Leu Pro Gly Asn Ile Ile Ser Ala Leu Leu Met		
545	550	555
Asp Arg Ile Gly Arg Leu Lys Met Ile Gly Gly Ser Met Leu Ile Ser		
565	570	575
Ala Val Cys Cys Phe Phe Leu Phe Phe Gly Asn Ser Glu Ser Ala Met		
580	585	590

115	120	125	
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cta ggc agc ata gtg tac ctc ggg atg atg gtg ggg gcg ttc ttc tgg Leu Gly Ser Ile Val Tyr Leu Gly Met Met Val Gly Ala Phe Phe Trp 195 200 205			624
gga gga ctg gca gac aaa gtg gga agg aaa cag tct ctt ctg att tgc Gly Gly Leu Ala Asp Lys Val Gly Arg Lys Gln Ser Leu Leu Ile Cys 210 215 220			672
atg tct gtc aac gga ttc ttt gcc ttc ctt tct tca ttt gtc caa ggt Met Ser Val Asn Gly Phe Phe Ala Phe Leu Ser Ser Phe Val Gln Gly 225 230 235 240			720
tat ggc ttc ttt ctc ttc tgt cgc tta ctt tct gga ttc ggg att gga Tyr Gly Phe Phe Leu Phe Cys Arg Leu Leu Ser Gly Phe Gly Ile Gly 245 250 255			768
gga gcc ata ccc act gtg ttc tcg tac ttt gct gaa gtc ctg gcc cgg Gly Ala Ile Pro Thr Val Phe Ser Tyr Phe Ala Glu Val Leu Ala Arg 260 265 270			816
gaa aag cgg ggc gaa cac ttg agc tgg ctc tgc atg ttc tgg atg atc Glu Lys Arg Gly Glu His Leu Ser Trp Leu Cys Met Phe Trp Met Ile 275 280 285			864
ggt ggc atc tac gcc tct gcc atg gcc tgg gcc atc atc ccg cac tac Gly Gly Ile Tyr Ala Ser Ala Met Ala Trp Ala Ile Ile Pro His Tyr 290 295 300			912
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gtg ttt gtc atc gtc tgt gca ctc ccc tgt gtc tcc tcc gtg gtg gcc Val Phe Val Ile Val Cys Ala Leu Pro Cys Val Ser Ser Val Val Ala 325 330 335			1008
ctc aca ttc atg cct gaa agc cca cga ttc ttg ttg gag gtt gga aaa Leu Thr Phe Met Pro Glu Ser Pro Arg Phe Leu Leu Glu Val Gly Lys 340 345 350			1056
cat gat gaa gct tgg atg att ctg aag tta att cat gac acc aac atg His Asp Glu Ala Trp Met Ile Leu Lys Leu Ile His Asp Thr Asn Met 355 360 365			1104

cgc tta aca atg cta ggt ggc tct atg gtg ctt tcg ggg atc agc tgt 1872
 Arg Leu Thr Met Leu Gly Gly Ser Met Val Leu Ser Gly Ile Ser Cys
 610 615 620

ttc ttc ctt tgg ttc ggc acc agt gaa tcc atg atg ata ggc atg ctg 1920
 Phe Phe Leu Trp Phe Gly Thr Ser Glu Ser Met Met Ile Gly Met Leu
 625 630 635 640

tgt ctg tac aat gga ttg acc atc tca gcc tgg aac tct ctt gac gtg 1968
 Cys Leu Tyr Asn Gly Leu Thr Ile Ser Ala Trp Asn Ser Leu Asp Val
 645 650 655

gtc act gtg gaa ctg tac ccc aca gac cgg agg gca aca ggc ttt ggc 2016
 Val Thr Val Glu Leu Tyr Pro Thr Asp Arg Arg Ala Thr Gly Phe Gly
 660 665 670

ttc tta aat gcg cta tgc aag gca gca gcc gtc ctg gga aac tta ata 2064
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 675 680 685

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 Phe Gly Ser Leu Val Ser Ile Thr Lys Ser Ile Pro Ile Leu Leu Ala
 690 695 700

tct act gtg ctc gtg tgt gga gga ctc gtt ggg ctg tgc ctg cct gac 2160
 Ser Thr Val Leu Val Cys Gly Gly Leu Val Gly Leu Cys Leu Pro Asp
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aca cga acc cag gtt ctg atg taa 2184
 Thr Arg Thr Gln Val Leu Met
 725

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 <212> PRT
 <213> Homo sapiens

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Ala Val Asp Arg Ala Gln Asp Glu Tyr Thr Gln Arg Ser Tyr Ser Arg
 35 40 45

Phe Gln Asp Glu Glu Asp Asp Asp Asp Tyr Tyr Pro Ala Gly Glu Thr
 50 55 60

Tyr Asn Gly Glu Ala Asn Asp Asp Glu Gly Ser Ser Glu Ala Thr Glu
 65 70 75 80

Gly His Asp Glu Asp Asp Glu Ile Tyr Glu Gly Glu Tyr Gln Gly Ile
 85 90 95

Pro Ser Met Asn Gln Ala Lys Asp Ser Ile Val Ser Val Gly Gln Pro
 100 105 110

Lys Gly Asp Glu Tyr Lys Asp Arg Arg Glu Leu Glu Ser Glu Arg Arg

Gly Leu Ser Val Trp Phe Pro Asp Val Ile Lys Pro Leu Gln Ser Asp
 450 455 460
 Glu Tyr Ala Leu Leu Thr Arg Asn Val Glu Arg Asp Lys Tyr Ala Asn
 465 470 475 480
 Phe Thr Ile Asn Phe Thr Met Glu Asn Gln Ile His Thr Gly Met Glu
 485 490 495
 Tyr Asp Asn Gly Arg Phe Ile Gly Val Lys Phe Lys Ser Val Thr Phe
 500 505 510
 Lys Asp Ser Val Phe Lys Ser Cys Thr Phe Glu Asp Val Thr Ser Val
 515 520 525
 Asn Thr Tyr Phe Lys Asn Cys Thr Phe Ile Asp Thr Val Phe Asp Asn
 530 535 540
 Thr Asp Phe Glu Pro Tyr Lys Phe Ile Asp Ser Glu Phe Lys Asn Cys
 545 550 555 560
 Ser Phe Phe His Asn Lys Thr Gly Cys Gln Ile Thr Phe Asp Asp Asp
 565 570 575
 Tyr Ser Ala Tyr Trp Ile Tyr Phe Val Asn Phe Leu Gly Thr Leu Ala
 580 585 590
 Val Leu Pro Gly Asn Ile Val Ser Ala Leu Leu Met Asp Arg Ile Gly
 595 600 605
 Arg Leu Thr Met Leu Gly Gly Ser Met Val Leu Ser Gly Ile Ser Cys
 610 615 620
 Phe Phe Leu Trp Phe Gly Thr Ser Glu Ser Met Met Ile Gly Met Leu
 625 630 635 640
 Cys Leu Tyr Asn Gly Leu Thr Ile Ser Ala Trp Asn Ser Leu Asp Val
 645 650 655
 Val Thr Val Glu Leu Tyr Pro Thr Asp Arg Arg Ala Thr Gly Phe Gly
 660 665 670
 Phe Leu Asn Ala Leu Cys Lys Ala Ala Ala Val Leu Gly Asn Leu Ile
 675 680 685
 Phe Gly Ser Leu Val Ser Ile Thr Lys Ser Ile Pro Ile Leu Leu Ala
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aag gca atc gcc acc tta aag agg ata gca act gaa aac gga gct ccc Lys Ala Ile Ala Thr Leu Lys Arg Ile Ala Thr Glu Asn Gly Ala Pro 275 280 285	864
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atg agg gac ctt ttc aca ccc cat ttt aga tgg aca act ttg ctg ctg Met Arg Asp Leu Phe Thr Pro His Phe Arg Trp Thr Thr Leu Leu Leu 305 310 315 320	960
tgg ttt ata tgg ttt tcc aat gca ttc tct tac tac ggg tta gtt cta Trp Phe Ile Trp Phe Ser Asn Ala Phe Ser Tyr Tyr Gly Leu Val Leu 325 330 335	1008
ctc acc aca gaa ctc ttc cag gca gga gat gtc tgc ggc atc tcc agt Leu Thr Thr Glu Leu Phe Gln Ala Gly Asp Val Cys Gly Ile Ser Ser 340 345 350	1056
cgg aag aag gct gta gag gca aaa tgc agc ctg gcc tgc gag tac ctg Arg Lys Lys Ala Val Glu Ala Lys Cys Ser Leu Ala Cys Glu Tyr Leu 355 360 365	1104
agt gag gag gat tac atg gac ttg ctg tgg acc acc ctc tct gag ttt Ser Glu Glu Asp Tyr Met Asp Leu Leu Trp Thr Thr Leu Ser Glu Phe 370 375 380	1152
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aag acc atg gcc ctg tgc ttt gtc atc ttc tcc ttc tgc agc ctc ctg Lys Thr Met Ala Leu Cys Phe Val Ile Phe Ser Phe Cys Ser Leu Leu 405 410 415	1248
ctg ttt atc tgt gtt gga aga aat gtg ctc act ctg tta ctc ttc att Leu Phe Ile Cys Val Gly Arg Asn Val Leu Thr Leu Leu Leu Phe Ile 420 425 430	1296
gca aga gcg ttt att tct gga ggc ttt caa gcg gca tat gtt tac aca Ala Arg Ala Phe Ile Ser Gly Gly Phe Gln Ala Ala Tyr Val Tyr Thr 435 440 445	1344
cct gag gtc tac ccc acg gca acg cgg gcc ctc ggc ctg ggc acc tgc Pro Glu Val Tyr Pro Thr Ala Thr Arg Ala Leu Gly Leu Gly Thr Cys 450 455 460	1392

Leu Tyr Tyr Gly Ile Leu Ser Ala Phe Ala Pro Val Tyr Ser Trp Ile
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 Leu Val Leu Arg Gly Leu Val Gly Phe Gly Ile Gly Gly Val Pro Gln
 180 185 190
 Ser Val Thr Leu Tyr Ala Glu Phe Leu Pro Met Lys Ala Arg Ala Lys
 195 200 205
 Cys Ile Leu Leu Ile Glu Val Phe Trp Ala Ile Gly Thr Val Phe Glu
 210 215 220
 Val Val Leu Ala Val Phe Val Met Pro Ser Leu Gly Trp Arg Trp Leu
 225 230 235 240
 Leu Ile Leu Ser Ala Val Pro Leu Leu Leu Phe Ala Val Leu Cys Phe
 245 250 255
 Trp Leu Pro Glu Ser Ala Arg Tyr Asp Val Leu Ser Gly Asn Gln Glu
 260 265 270
 Lys Ala Ile Ala Thr Leu Lys Arg Ile Ala Thr Glu Asn Gly Ala Pro
 275 280 285
 Met Pro Leu Gly Lys Leu Ile Ile Ser Arg Gln Glu Asp Arg Gly Lys
 290 295 300
 Met Arg Asp Leu Phe Thr Pro His Phe Arg Trp Thr Thr Leu Leu Leu
 305 310 315 320
 Trp Phe Ile Trp Phe Ser Asn Ala Phe Ser Tyr Tyr Gly Leu Val Leu
 325 330 335
 Leu Thr Thr Glu Leu Phe Gln Ala Gly Asp Val Cys Gly Ile Ser Ser
 340 345 350
 Arg Lys Lys Ala Val Glu Ala Lys Cys Ser Leu Ala Cys Glu Tyr Leu
 355 360 365
 Ser Glu Glu Asp Tyr Met Asp Leu Leu Trp Thr Thr Leu Ser Glu Phe
 370 375 380
 Pro Gly Val Leu Val Thr Leu Trp Ile Ile Asp Arg Leu Gly Arg Lys
 385 390 395 400
 Lys Thr Met Ala Leu Cys Phe Val Ile Phe Ser Phe Cys Ser Leu Leu
 405 410 415
 Leu Phe Ile Cys Val Gly Arg Asn Val Leu Thr Leu Leu Leu Phe Ile
 420 425 430
 Ala Arg Ala Phe Ile Ser Gly Gly Phe Gln Ala Ala Tyr Val Tyr Thr
 435 440 445
 Pro Glu Val Tyr Pro Thr Ala Thr Arg Ala Leu Gly Leu Gly Thr Cys
 450 455 460
 Ser Gly Met Ala Arg Val Gly Ala Leu Ile Thr Pro Phe Ile Ala Gln
 465 470 475 480
 Val Met Leu Glu Ser Ser Val Tyr Leu Thr Leu Ala Val Tyr Ser Gly

ggc cac gat gag gat gat gag atc tac gag gga gaa tat cag ggc atc Gly His Asp Glu Asp Asp Glu Ile Tyr Glu Gly Glu Tyr Gln Gly Ile 90 95 100	702
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ccc ctg gct gga gtg aga ggg ggc tta agt gat ggg gag ggt ccc cct Pro Leu Ala Gly Val Arg Gly Gly Leu Ser Asp Gly Glu Gly Pro Pro 120 125 130	798
ggg ggt cgc ggg gag gcg cag cgg cgt aaa gat cgg gaa gaa ttg gct Gly Gly Arg Gly Glu Ala Gln Arg Arg Lys Asp Arg Glu Glu Leu Ala 135 140 145	846
cag cag tat gag acc atc ctc cgg gag tgc ggc cat ggt cgc ttc cag Gln Gln Tyr Glu Thr Ile Leu Arg Glu Cys Gly His Gly Arg Phe Gln 150 155 160 165	894
tgg aca ctc tac ttc gtg ctg ggt ctg gcg ctg atg gcc gat ggt gta Trp Thr Leu Tyr Phe Val Leu Gly Leu Ala Leu Met Ala Asp Gly Val 170 175 180	942
gag gtc ttt gtg gtg ggc ttt gtg ctg ccc agt gct gag aaa gat atg Glu Val Phe Val Val Gly Phe Val Leu Pro Ser Ala Glu Lys Asp Met 185 190 195	990
tgc ctg tcg gac tcc aac aaa ggc atg cta ggc ctc att gtg tac ctg Cys Leu Ser Asp Ser Asn Lys Gly Met Leu Gly Leu Ile Val Tyr Leu 200 205 210	1038
ggc atg atg gtg ggg gcc ttc ctc tgg gga ggc ctg gct gat cgg ctg Gly Met Met Val Gly Ala Phe Leu Trp Gly Gly Leu Ala Asp Arg Leu 215 220 225	1086
ggg cgg aga cag tgt ctg ctc atc tcg ctc tca gtc aac agc gtc ttc Gly Arg Arg Gln Cys Leu Leu Ile Ser Leu Ser Val Asn Ser Val Phe 230 235 240 245	1134
gct ttc ttc tca tcc ttc gtc cag ggt tat ggc acc ttc ctt ttc tgc Ala Phe Phe Ser Ser Phe Val Gln Gly Tyr Gly Thr Phe Leu Phe Cys 250 255 260	1182
cgc ctc ctt tct ggg gtt ggg att ggt ggt tcc atc ccc att gtc ttc Arg Leu Leu Ser Gly Val Gly Ile Gly Gly Ser Ile Pro Ile Val Phe 265 270 275	1230
tcc tat ttt tcg gag ttt ctg gcc cag gag aaa cgt ggg gag cat ttg Ser Tyr Phe Ser Glu Phe Leu Ala Gln Glu Lys Arg Gly Glu His Leu 280 285 290	1278
agc tgg ctc tgt atg ttc tgg atg att ggt ggc gtg tat gca gct gca Ser Trp Leu Cys Met Phe Trp Met Ile Gly Gly Val Tyr Ala Ala Ala 295 300 305	1326
atg gcc tgg gcc atc atc ccc cac tat ggg tgg agt ttc cag atg ggc Met Ala Trp Ala Ile Ile Pro His Tyr Gly Trp Ser Phe Gln Met Gly 310 315 320 325	1374

25/44

Phe Val Asn Ser Arg Leu Val Asn Ser Thr Phe Leu His Asn Lys Glu	
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ggt tgc cca cta gat gtg aca ggg acg ggc gaa ggt gcc tac atg gtg	2190
Gly Cys Pro Leu Asp Val Thr Gly Thr Gly Glu Gly Ala Tyr Met Val	
585 590 595	
tac ttt gtc agc ttc ttg ggg aca ctg gct gtg ctc cct gga aat att	2238
Tyr Phe Val Ser Phe Leu Gly Thr Leu Ala Val Leu Pro Gly Asn Ile	
600 605 610	
gtg tct gct ctg ctc atg gac aag att ggc agg ctc aga atg ctt gct	2286
Val Ser Ala Leu Leu Met Asp Lys Ile Gly Arg Leu Arg Met Leu Ala	
615 620 625	
ggt tcc agt gtg ttg tcc tgt gtt tcc tgc ttc ttc ctg tct ttt ggg	2334
Gly Ser Ser Val Leu Ser Cys Val Ser Cys Phe Phe Leu Ser Phe Gly	
630 635 640 645	
aac agt gag tca gcc atg atc gct ctg ctc tgc ctt ttt ggg gga gtc	2382
Asn Ser Glu Ser Ala Met Ile Ala Leu Leu Cys Leu Phe Gly Gly Val	
650 655 660	
agt att gca tcc tgg aac gcg ctg gac gtg ctg act gtt gaa ctc tac	2430
Ser Ile Ala Ser Trp Asn Ala Leu Asp Val Leu Thr Val Glu Leu Tyr	
665 670 675	
cct tcc gac aag agg acg acg gcc ttc ggc ttc ctg aat gcc ctg tgt	2478
Pro Ser Asp Lys Arg Thr Thr Ala Phe Gly Phe Leu Asn Ala Leu Cys	
680 685 690	
aag ctg gca gct gta ctg ggc atc agc atc ttc acg tcc ttt gtg gga	2526
Lys Leu Ala Ala Val Leu Gly Ile Ser Ile Phe Thr Ser Phe Val Gly	
695 700 705	
atc acc aag gcc gct ccc atc ctc ttc gcc tca gct gcg ctt gcc ctt	2574
Ile Thr Lys Ala Ala Pro Ile Leu Phe Ala Ser Ala Ala Leu Ala Leu	
710 715 720 725	
ggt agc tct ctg gct ctg aag ctg cct gag acc cgg gga cag gtg ctg	2622
Gly Ser Ser Leu Ala Leu Lys Leu Pro Glu Thr Arg Gly Gln Val Leu	
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Gln	
caataatttc ttttatecct accctgccct gctgtcctgg tectactcgg tgtttggtgt	2738
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ctcaattcag gctacaagtg tgaagcctgg attttatgga attagttttc tgattcttat	3158

His Gly Arg Phe Gln Trp Thr Leu Tyr Phe Val Leu Gly Leu Ala Leu
 165 170 175
 Met Ala Asp Gly Val Glu Val Phe Val Val Gly Phe Val Leu Pro Ser
 180 185 190
 Ala Glu Lys Asp Met Cys Leu Ser Asp Ser Asn Lys Gly Met Leu Gly
 195 200 205
 Leu Ile Val Tyr Leu Gly Met Met Val Gly Ala Phe Leu Trp Gly Gly
 210 215 220
 Leu Ala Asp Arg Leu Gly Arg Arg Gln Cys Leu Leu Ile Ser Leu Ser
 225 230 235 240
 Val Asn Ser Val Phe Ala Phe Phe Ser Ser Phe Val Gln Gly Tyr Gly
 245 250 255
 Thr Phe Leu Phe Cys Arg Leu Leu Ser Gly Val Gly Ile Gly Gly Ser
 260 265 270
 Ile Pro Ile Val Phe Ser Tyr Phe Ser Glu Phe Leu Ala Gln Glu Lys
 275 280 285
 Arg Gly Glu His Leu Ser Trp Leu Cys Met Phe Trp Met Ile Gly Gly
 290 295 300
 Val Tyr Ala Ala Ala Met Ala Trp Ala Ile Ile Pro His Tyr Gly Trp
 305 310 315 320
 Ser Phe Gln Met Gly Ser Ala Tyr Gln Phe His Ser Trp Arg Val Phe
 325 330 335
 Val Leu Val Phe Ala Phe Pro Ser Val Phe Ala Ile Gly Ala Leu Thr
 340 345 350
 Thr Gln Pro Glu Ser Pro Arg Phe Phe Leu Glu Asn Gly Lys His Asp
 355 360 365
 Glu Ala Trp Met Val Leu Lys Gln Val His Asp Thr Asn Met Arg Ala
 370 375 380
 Lys Gly His Pro Glu Arg Val Phe Ser Val Thr His Ile Lys Thr Ile
 385 390 395 400
 His Gln Glu Asp Glu Leu Ile Glu Ile Gln Ser Asp Thr Gly Thr Trp
 405 410 415
 Tyr Gln Arg Trp Gly Val Arg Ala Leu Ser Leu Gly Gly Gln Val Trp
 420 425 430
 Gly Asn Phe Leu Ser Cys Phe Ser Pro Glu Tyr Arg Arg Ile Thr Leu
 435 440 445
 Met Met Met Gly Val Trp Phe Thr Met Ser Phe Ser Tyr Tyr Gly Leu
 450 455 460
 Thr Val Trp Phe Pro Asp Met Ile Arg His Leu Gln Ala Val Asp Tyr
 465 470 475 480

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 Asp Gly Tyr Tyr Arg Gly Asn Glu Gln Asn Pro Glu Glu Asp Ala Gln
 20 25 30

agc gat gtt aca gaa ggc cac gat gaa gag gat gag atc tat gag ggc 144
 Ser Asp Val Thr Glu Gly His Asp Glu Glu Asp Glu Ile Tyr Glu Gly
 35 40 45

gag tac caa ggc atc cct cat cca gat gat gtc aag tct aag cag act 192
 Glu Tyr Gln Gly Ile Pro His Pro Asp Asp Val Lys Ser Lys Gln Thr
 50 55 60

aag atg gca ccg tcc aga gca gat ggc ctt cgg ggc cag gca gac ctg 240
 Lys Met Ala Pro Ser Arg Ala Asp Gly Leu Arg Gly Gln Ala Asp Leu
 65 70 75 80

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 Met Ala Glu Arg Met Glu Asp Glu Glu Gln Leu Ala His Gln Tyr Glu
 85 90 95

acc atc att gat gag tgt ggc cat ggg cgc ttc cag tgg acc ctc ttt 336
 Thr Ile Ile Asp Glu Cys Gly His Gly Arg Phe Gln Trp Thr Leu Phe
 100 105 110

ttc gtc ttg gtc ttg gcc ttg atg gct gac gga gtg gaa gtg ttt gtg 384
 Phe Val Leu Val Leu Ala Leu Met Ala Asp Gly Val Glu Val Phe Val
 115 120 125

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 Val Ser Phe Ala Leu Pro Ser Ala Glu Lys Asp Met Cys Leu Ser Ser
 130 135 140

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 Ser Lys Lys Gly Met Leu Gly Leu Ile Val Tyr Leu Gly Met Met Ala
 145 150 155 160

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 Gly Ala Phe Ile Leu Gly Gly Leu Ala Asp Lys Leu Gly Arg Lys Lys
 165 170 175

gtc ctc agc atg tcc ttg gct atc aat gct tcc ttt gcc tcc ctc tcc 576
 Val Leu Ser Met Ser Leu Ala Ile Asn Ala Ser Phe Ala Ser Leu Ser
 180 185 190

tcc ttc gtg cag gga tat gga gct ttc ctc ttc tgc aga ctc atc tca 624
 Ser Phe Val Gln Gly Tyr Gly Ala Phe Leu Phe Cys Arg Leu Ile Ser
 195 200 205

ggc ata ggt att ggg ggc tcc ctg cca att gtt ttt gcc tac ttt tct 672
 Gly Ile Gly Ile Gly Gly Ser Leu Pro Ile Val Phe Ala Tyr Phe Ser
 210 215 220

gag ttc tta tca cgg gag aaa cgc ggt gag cat ctc agc tgg ctg ggt 720
 Glu Phe Leu Ser Arg Glu Lys Arg Gly Glu His Leu Ser Trp Leu Gly
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31/44

gat gtg aca tcc aca gat act tat ttc aag aac tgc acc att gaa tcg 1488
Asp Val Thr Ser Thr Asp Thr Tyr Phe Lys Asn Cys Thr Ile Glu Ser
485 490 495

act acc ttc tac aac aca gac ctc tac aaa cac aag ttc att gac tgt 1536
Thr Thr Phe Tyr Asn Thr Asp Leu Tyr Lys His Lys Phe Ile Asp Cys
500 505 510

cgg ttt atc aat tcc acc ttt ctg gag cag aag gag ggc tgc cac atg 1584
Arg Phe Ile Asn Ser Thr Phe Leu Glu Gln Lys Glu Gly Cys His Met
515 520 525

gac ttt gaa gag gac aat gat ttt ctg att tac ctc gtc agc ttc ctc 1632
Asp Phe Glu Glu Asp Asn Asp Phe Leu Ile Tyr Leu Val Ser Phe Leu
530 535 540

ggc agc ctg tct gtc ttg cct ggg aac ata att tct gcc ctg ctc atg 1680
Gly Ser Leu Ser Val Leu Pro Gly Asn Ile Ile Ser Ala Leu Leu Met
545 550 555 560

gac aga atc gga aga ctt aag atg att ggt ggc tcc atg ctc atc tct 1728
Asp Arg Ile Gly Arg Leu Lys Met Ile Gly Gly Ser Met Leu Ile Ser
565 570 575

gca gtc tgc tgc ttc ttc ctg ttt ttt ggc aac agc gag tct gcg atg 1776
Ala Val Cys Cys Phe Phe Leu Phe Phe Gly Asn Ser Glu Ser Ala Met
580 585 590

atc ggc tgg caa tgc ctg ttc tgt ggg acc agc att gca gcc tgg aat 1824
Ile Gly Trp Gln Cys Leu Phe Cys Gly Thr Ser Ile Ala Ala Trp Asn
595 600 605

gct ctg gat gtg atc aca gta gag ctg tat ccc acc aac cag agg gcc 1872
Ala Leu Asp Val Ile Thr Val Glu Leu Tyr Pro Thr Asn Gln Arg Ala
610 615 620

act gcc ttc ggc atc ctc aat gga ctg tgc aaa ctt ggt gcc atc ctg 1920
Thr Ala Phe Gly Ile Leu Asn Gly Leu Cys Lys Leu Gly Ala Ile Leu
625 630 635 640

gga aac act atc ttt gct tct ttt gtt ggg atc acc aaa gtg gtc ccc 1968
Gly Asn Thr Ile Phe Ala Ser Phe Val Gly Ile Thr Lys Val Val Pro
645 650 655

atc ctt ctg gct gct gct tct ctg gtt gga ggt ggc ttg gtt gcc ctt 2016
Ile Leu Leu Ala Ala Ala Ser Leu Val Gly Gly Gly Leu Val Ala Leu
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cga ctg cca gag act cga gag cag gtc ctg atg tga 2052
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<212> PRT

<213> Rattus norvegicus

<400> 12

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 355 360 365
 Thr Ile Phe Lys Gln Val Trp Asp Asn Ala Leu Tyr Cys Val Met Gly
 370 375 380
 Pro Tyr Arg Met Asn Thr Leu Ile Leu Ala Val Val Trp Phe Thr Met
 385 390 395 400
 Ala Leu Ser Tyr Tyr Gly Leu Thr Val Trp Phe Pro Asp Met Ile Arg
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 Tyr Phe Gln Asp Glu Glu Tyr Lys Ser Lys Met Lys Val Phe Phe Gly
 420 425 430
 Glu His Val His Gly Ala Thr Ile Asn Phe Thr Met Glu Asn Gln Ile
 435 440 445
 His Gln His Gly Lys Leu Val Asn Asp Lys Phe Ile Lys Met Tyr Phe
 450 455 460
 Lys His Val Leu Phe Glu Asp Thr Phe Phe Asp Lys Cys Tyr Phe Glu
 465 470 475 480
 Asp Val Thr Ser Thr Asp Thr Tyr Phe Lys Asn Cys Thr Ile Glu Ser
 485 490 495
 Thr Thr Phe Tyr Asn Thr Asp Leu Tyr Lys His Lys Phe Ile Asp Cys
 500 505 510
 Arg Phe Ile Asn Ser Thr Phe Leu Glu Gln Lys Glu Gly Cys His Met
 515 520 525
 Asp Phe Glu Glu Asp Asn Asp Phe Leu Ile Tyr Leu Val Ser Phe Leu
 530 535 540
 Gly Ser Leu Ser Val Leu Pro Gly Asn Ile Ile Ser Ala Leu Leu Met
 545 550 555 560
 Asp Arg Ile Gly Arg Leu Lys Met Ile Gly Gly Ser Met Leu Ile Ser
 565 570 575
 Ala Val Cys Cys Phe Phe Leu Phe Phe Gly Asn Ser Glu Ser Ala Met
 580 585 590
 Ile Gly Trp Gln Cys Leu Phe Cys Gly Thr Ser Ile Ala Ala Trp Asn
 595 600 605
 Ala Leu Asp Val Ile Thr Val Glu Leu Tyr Pro Thr Asn Gln Arg Ala
 610 615 620
 Thr Ala Phe Gly Ile Leu Asn Gly Leu Cys Lys Leu Gly Ala Ile Leu
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 Gly Asn Thr Ile Phe Ala Ser Phe Val Gly Ile Thr Lys Val Val Pro
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 Ile Leu Leu Ala Ala Ala Ser Leu Val Gly Gly Gly Leu Val Ala Leu

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gac ggc gtg gag gtg ttt gtg gtg ggc ttt gtg tta ccc agt gca gag Asp Gly Val Glu Val Phe Val Val Gly Phe Val Leu Pro Ser Ala Glu 165 170 175 180			763
aca gac cta tgc ata ccg aat tca gga tct gga tgg cta ggc agc ata Thr Asp Leu Cys Ile Pro Asn Ser Gly Ser Gly Trp Leu Gly Ser Ile 185 190 195			811
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ccc gaa agc cct cgg ttc ttg ctg gag gta gga aaa cat gat gaa gcc Pro Glu Ser Pro Arg Phe Leu Leu Glu Val Gly Lys His Asp Glu Ala 345 350 355			1291
tgg atg att ctg aag cta att cat gat acc aac atg aga gcc cgg ggc Trp Met Ile Leu Lys Leu Ile His Asp Thr Asn Met Arg Ala Arg Gly 360 365 370			1339
cag cca gag aag gtc ttc acg gta aat aaa atc aag act ccc aag caa Gln Pro Glu Lys Val Phe Thr Val Asn Lys Ile Lys Thr Pro Lys Gln 375 380 385			1387

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gga ctg acc atc tca gcg tgg aac tct ctt gat gta gtc acg gtg gaa 2203
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 645 650 655 660

cta tac ccc aca gac cgg aga gca acg ggc ttt ggc ttc ttg aac gca 2251
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 665 670 675

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 Val Ser Ile Thr Lys Ala Ile Pro Ile Leu Leu Ala Ser Thr Val Leu
 695 700 705

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 Val Cys Gly Gly Leu Val Gly Leu Arg Leu Pro Asp Thr Arg Thr Gln
 710 715 720

gtt ctg atg tga caaaagccat tctcttctct caccatgggt cagccctatt 2447
 Val Leu Met
 725

gcctgactca aggcttcaga gtttttatgt atagaaaggt ggccaagtat cagaactcaa 2507

acttttgctg tgacgtaa at gtagctgtgt attgtccccg ccagtgtgat ttgcagggtc 2567

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 <213> Rattus norvegicus

<400> 14

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Ala Val Asp Arg Ala Gln Asp Glu Tyr Thr Gln Arg Ser Tyr Ser Arg
 35 40 45

Phe Gln Asp Glu Asp Asp Asp Asp Tyr Tyr Pro Pro Gly Glu Thr
 50 55 60

Tyr Ser Gly Glu Ala Asn Asp Asp Glu Gly Ser Ser Glu Ala Thr Glu
 65 70 75 80

Gly His Asp Glu Glu Asp Glu Ile Tyr Glu Gly Glu Tyr Gln Gly Ile
 85 90 95

Pro Ser Thr Asn Gln Gly Lys Asp Ser Ile Val Ser Val Gly Gln Pro
 100 105 110

Thr Ile Lys Leu Thr Ile Val Trp Phe Thr Leu Ser Phe Gly Tyr Tyr
 435 440 445
 Gly Leu Ser Val Trp Phe Pro Asp Val Ile Lys His Leu Gln Ser Asp
 450 455 460
 Glu Tyr Ala Leu Leu Thr Arg Asn Val Gln Lys Asp Lys Tyr Ala Asn
 465 470 475 480
 Phe Ser Ile Asn Phe Thr Met Glu Asn Gln Val His Thr Gly Met Glu
 485 490 495
 Tyr Asp Asn Gly Arg Phe Leu Gly Val Lys Phe Lys Ser Val Thr Phe
 500 505 510
 Lys Asp Ser Val Phe Lys Ser Cys Thr Phe Asp Asp Val Thr Ser Val
 515 520 525
 Asn Thr Tyr Phe Lys Asn Cys Thr Phe Ile Asp Thr Leu Phe Glu Asn
 530 535 540
 Thr Asp Phe Glu Pro Tyr Lys Phe Ile Asp Ser Glu Phe Gln Asn Cys
 545 550 555 560
 Ser Phe Leu His Asn Lys Thr Gly Cys Gln Ile Thr Phe Asp Asp Asp
 565 570 575
 Tyr Ser Ala Tyr Trp Ile Tyr Phe Val Asn Phe Leu Gly Thr Leu Ala
 580 585 590
 Val Leu Pro Gly Asn Ile Val Ser Ala Leu Leu Met Asp Arg Ile Gly
 595 600 605
 Arg Leu Thr Met Leu Gly Gly Ser Met Val Leu Ser Gly Ile Ser Cys
 610 615 620
 Phe Phe Leu Trp Phe Gly Thr Ser Glu Ser Met Met Ile Gly Met Leu
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 Cys Leu Tyr Asn Gly Leu Thr Ile Ser Ala Trp Asn Ser Leu Asp Val
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 Val Thr Val Glu Leu Tyr Pro Thr Asp Arg Arg Ala Thr Gly Phe Gly
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 Phe Leu Asn Ala Leu Cys Lys Ala Ala Ala Val Leu Gly Asn Leu Ile
 675 680 685
 Phe Gly Ser Leu Val Ser Ile Thr Lys Ala Ile Pro Ile Leu Leu Ala
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Leu Leu Leu Ser Ala Ala Pro Leu Leu Val Phe Ala Val Leu Cys Phe	
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tgg ctg cca gag agt gct agg tac gat gtg ctg tct ggg aac cag gaa	816
Trp Leu Pro Glu Ser Ala Arg Tyr Asp Val Leu Ser Gly Asn Gln Glu	
260 265 270	
aag gcg att gct acc tta aag cgg atc gca aca gaa aat gga gcc ccc	864
Lys Ala Ile Ala Thr Leu Lys Arg Ile Ala Thr Glu Asn Gly Ala Pro	
275 280 285	
atg cct ctg ggg aag ctc atc atc tcc aga cag gaa gac cga ggc aaa	912
Met Pro Leu Gly Lys Leu Ile Ile Ser Arg Gln Glu Asp Arg Gly Lys	
290 295 300	
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Met Arg Asp Leu Phe Thr Pro His Phe Arg Trp Thr Thr Leu Leu Leu	
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Trp Phe Ile Trp Phe Ser Asn Ala Phe Ser Tyr Tyr Gly Leu Val Leu	
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Leu Thr Thr Glu Leu Phe Gln Ala Gly Asp Val Cys Ser Ile Ser Ser	
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Arg Lys Lys Ala Val Glu Ala Lys Cys Ser Leu Ala Cys Glu Tyr Leu	
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Ser Lys Glu Asp Tyr Met Asp Leu Leu Trp Thr Thr Leu Ser Glu Phe	
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Pro Gly Val Leu Val Thr Leu Trp Val Ile Asp Arg Leu Gly Arg Lys	
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Lys Thr Met Ala Leu Cys Phe Val Ile Phe Ser Leu Cys Ser Leu Leu	
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cct gag gtg tat cca acg gcg acg agg gcg ctg ggc ctg ggc acc tgc	1392
Pro Glu Val Tyr Pro Thr Ala Thr Arg Ala Leu Gly Leu Gly Thr Cys	

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Leu Tyr Tyr Gly Ile Leu Ser Ala Phe Ala Pro Val Tyr Ser Trp Ile	165	170	175
Leu Val Leu Arg Gly Leu Val Gly Phe Gly Ile Gly Gly Val Pro Gln	180	185	190
Ser Val Thr Leu Tyr Ala Glu Phe Leu Pro Met Lys Ala Arg Ala Lys	195	200	205
Cys Ile Leu Leu Ile Glu Val Phe Trp Ala Ile Gly Thr Val Phe Glu	210	215	220
Val Leu Leu Ala Val Phe Val Met Pro Ser Leu Gly Trp Arg Trp Leu	225	230	235
Leu Leu Leu Ser Ala Ala Pro Leu Leu Val Phe Ala Val Leu Cys Phe	245	250	255
Trp Leu Pro Glu Ser Ala Arg Tyr Asp Val Leu Ser Gly Asn Gln Glu	260	265	270
Lys Ala Ile Ala Thr Leu Lys Arg Ile Ala Thr Glu Asn Gly Ala Pro	275	280	285
Met Pro Leu Gly Lys Leu Ile Ile Ser Arg Gln Glu Asp Arg Gly Lys	290	295	300
Met Arg Asp Leu Phe Thr Pro His Phe Arg Trp Thr Thr Leu Leu Leu	305	310	315
Trp Phe Ile Trp Phe Ser Asn Ala Phe Ser Tyr Tyr Gly Leu Val Leu	325	330	335
Leu Thr Thr Glu Leu Phe Gln Ala Gly Asp Val Cys Ser Ile Ser Ser	340	345	350
Arg Lys Lys Ala Val Glu Ala Lys Cys Ser Leu Ala Cys Glu Tyr Leu	355	360	365
Ser Lys Glu Asp Tyr Met Asp Leu Leu Trp Thr Thr Leu Ser Glu Phe	370	375	380
Pro Gly Val Leu Val Thr Leu Trp Val Ile Asp Arg Leu Gly Arg Lys	385	390	395
Lys Thr Met Ala Leu Cys Phe Val Ile Phe Ser Leu Cys Ser Leu Leu	405	410	415
Leu Phe Ile Cys Ile Gly Arg Asn Val Leu Thr Leu Leu Leu Phe Ile	420	425	430
Ala Arg Ala Phe Ile Ser Gly Gly Phe Gln Ala Ala Tyr Val Tyr Thr	435	440	445
Pro Glu Val Tyr Pro Thr Ala Thr Arg Ala Leu Gly Leu Gly Thr Cys	450	455	460
Ser Gly Met Ala Arg Val Gly Ala Leu Ile Thr Pro Phe Ile Ala Gln	465	470	475
			480

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